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Vaccinia virus encodes at least eight proteins that are modified post-translationally by the addition of a 16-carbon saturated fatty acid through thioester linkage to cysteine residues. This is referred to as palmitylation of proteins. The purpose of this work was to gain an understanding of palmitylation, focusing on what defined the substrate for the modification, and the biological relevance of protein palmitylation in the vaccinia virus life cycle.

A systematic approach was taken to identify the genes in vaccinia virus that encode these proteins. We found that vaccinia virus palmitylproteins are of the "late" temporal class, associate with intracellular membranes, and are specific for a particular form of the infectious virion. These criteria were used to narrow the number of genes expressed by vaccinia virus that potentially encode palmitylproteins. The "candidate" palmitylprotein genes were cloned and transiently expressed in mammalian tissue culture cells and analyzed for incorporation of palmitic acid. In addition to three previously identified vaccinia virus palmitylproteins, three new palmitylproteins were identified. The six known palmitylprotein genes were mutated to determine the site of modification, leading to the identification of the modification site for four of the six proteins.

One of the proteins, p37, was analyzed further for biological significance of the palmitate modification. A recombinant

vaccinia virus was constructed that did not express the wild-type palmitylated form of p37, but expressed a nonpalmitylated form of the protein instead. This virus was severely inhibited from proceeding past a particular morphogenetic stage, leading to an attenuated phenotype in tissue culture systems. Although the expression of the nonpalmitylated protein appeared normal compared to the wild-type protein, the lack of the palmityl moiety resulted in the loss of a targeting signal that directed the protein to its normal intracellular location.

By this work, significant contributions have been made toward understanding the process of protein palmitylation. We have identified, at least for vaccinia virus, primary structural determinants specifying the modification, leading to the identification of a palmitylation motif. Considering the attenuated phenotype of the mutant virus, our conclusion is that palmitylation is necessary for biological function, at least for p37.

Palmitylation of Vaccinia Virus Proteins: Identification of Modification Sites and Biological Relevance

by

Douglas W. Grosenbach

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Douglas W. Grosenbach

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CONTRIBUTION OF AUTHORS

Scott Hansen provided intellectual input and technical assistance for the work described in Chapter 2. All the work described in this thesis was performed in the laboratory of Dr. Dennis Hruby. He advised me on the identification the scientific problems addressed herein as well as the steps to be taken towards their solution.

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PREFACE

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Palmitylation of Vaccinia Virus Proteins: Identification of Modification Sites and Biological Relevance

Chapter 1

Introduction: Biology of Vaccinia Virus Acylproteins

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1.1 Introduction

Post-translational processing of vaccinia virus proteins has been proven to be a common mechanism for exerting regulatory control of function or targeting to subcellular and/or subviral structures. Fatty acylation, most commonly observed as the addition of myristate or palmitate, occurs on numerous vaccinia proteins and affects each in a distinct manner. Labeling of vaccinia-infected cells with tritiated myristic or palmitic acids demonstrates that vaccinia encodes at least six myristylproteins and as many as eight palmitylproteins. Where investigated, each of these proteins has been demonstrated to play important roles in the virus life cycle. Likewise, in each case studied, the fatty acyl modification greatly influences the function and/or biological activity of the protein.

1.1.1 Overview of the Vaccinia Life Cycle

Vaccinia virus (VV) is the most extensively characterized member of the Orthopoxvirus genus of the *Poxviridae* family (61). Its role as a vaccine in the eradication of smallpox represents one of the major achievements in medicine. Although smallpox vaccines are no longer necessary, VV is widely used as a molecular and cellular biology tool as well as in the development of recombinant vaccines. To enhance or optimize VV for these purposes, basic research on the virus itself remains a major area of interest. Current research topics include structure/morphogenesis, replication/resolution of the genome, transcriptional regulation, enzymology, immune modulation, and protein processing.

VV is a large, double-stranded DNA virus with a broad mammalian host range. Its entire life cycle is carried out in the

cytoplasm of the host, with little or no requirement for the host cell nucleus (Figure 1.1). Upon entry into the cell, the virion core is partially uncoated, allowing transcription of the early class of genes. Their products are involved in genome replication and as transcription factors for the intermediate class of genes. The VV genome is composed of a nearly 200 kilobasepair linear double-stranded DNA molecule with potential to encode more than 200 polypeptides (32).

VV open reading frames are designated alphanumerically. Digestion of VV genomic DNA with the restriction enzyme *Hin*dIII generates sixteen fragments, named fragments "A" through "P", with "A" being the largest. Open reading frames within each fragment are numbered from "left to right". Finally, each open reading frame is given an "R" or "L" to indicate the direction of transcription relative to the "left" end of the fragment. For example, the open reading frame F13L is encoded by the sixth-largest fragment (F). It is the thirteenth open reading frame from the left end of the fragment and is transcribed toward the left end of the fragment.

Host protein synthesis is rapidly and efficiently inhibited upon infection so that nearly the entire translational capacity of a cell is harnessed by the virus. Following intermediate gene expression and genome replication the late class of genes are expressed. The majority of the late gene products are involved in the late stages of virion development serving as structural components or scaffolding for nascent virions. Virion morphogenesis occurs in perinuclear macromolecular clusters containing numerous copies of the viral genome and virus encoded proteins referred to as virus factories, viroplasm or virosomes. Immature virions form by packaging viroplasm within membrane crescents most likely derived from the membranes of the intermediate compartment (93) but possibly produced *de novo* by virus-specific membrane forming machinery (41). The genome is packaged within these membrane crescents in the proteinaceous

core with the virus-encoded RNA polymerase and numerous cofactors involved in the early stages of the virus life cycle. Concomitant with the proteolytic processing of the three major core proteins (104), the core condenses, producing the first infectious form of the virus, which is referred to as intracellular mature virus (IMV). At this stage the core is wrapped with one proteolipid envelope.

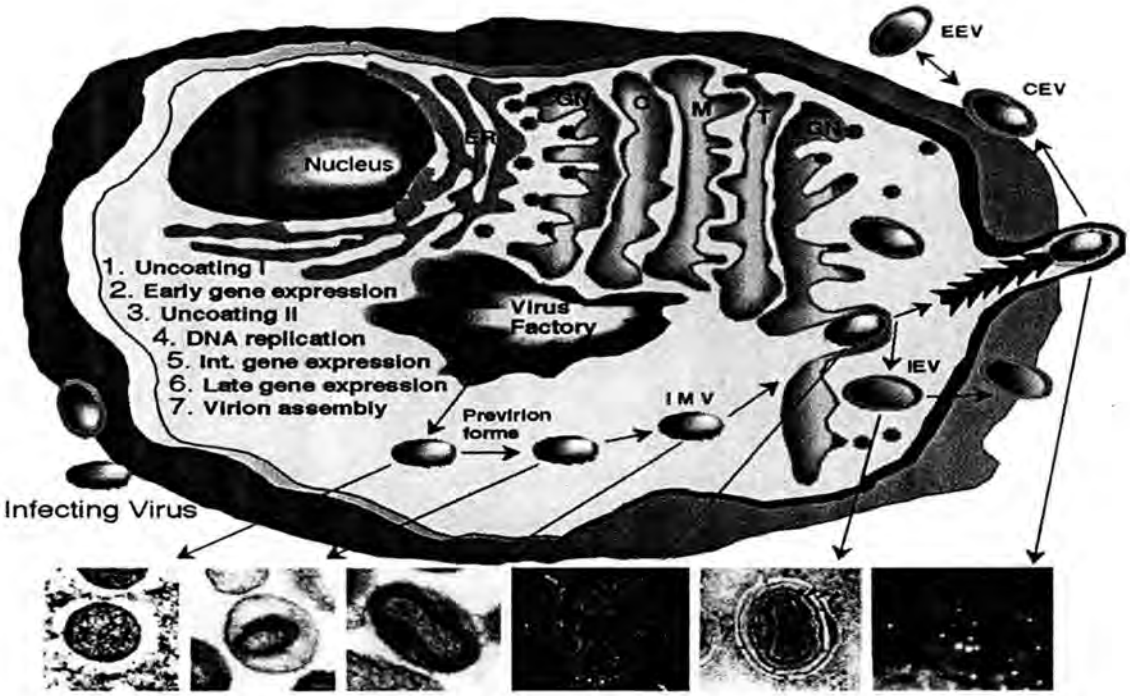

Figure 1.1. The VV replication cycle. A diagram of the infected cell is shown with an exaggerated view of the endoplasmic reticulum (ER), *cis*, *medial*, *trans* Golgi and the *trans*-Golgi network (C, M, T, and TGN respectively). The major stages of the virus life cycle are listed. Following late gene expression, previrion forms assemble to form intracellular mature virus (IMV). The IMV is targeted to the TGN and following envelopment, intracellular enveloped virus (IEV) is formed. IEV are propelled to the cell surface by the polymerization of actin filaments. Once released the virus may remain attached to the membrane as cell-associated enveloped virus (CEV) or be released into the medium as extracellular enveloped virus (EEV).

The IMV particle is targeted to the *trans*-Golgi network (TGN) and by budding through the compartment acquires two additional membranes (38). After envelopment by TGN membranes, the triple membrane-bound particle referred to as intracellular enveloped virus (IEV) is propelled to the cell surface by the formation of thick actin filaments behind it (17). At the cell surface the outermost virion membrane may be lost by fusion with the cell membrane, resulting in the release of a double membrane-bound particle referred to as extracellular enveloped virus (EEV). If the virion remains attached or reattaches to the cell surface, it is referred to as cell-associated enveloped virus (CEV). Some poxviruses also package virions in cytoplasmic inclusion bodies referred to as A-type inclusions (ATI), which are primarily composed of a single protein, the ATI protein (63). The ATI protein is truncated and correspondingly nonfunctional in VV so that no inclusion bodies form although the truncated form of the ATI protein is still expressed at high levels (19).

The enveloped forms of the virus (IEV, EEV, and CEV) are antigenically distinct from the IMV particle in that they contain at least six proteins in their outer envelope(s) that are not present on IMV. They are encoded by the A33R (81), A34R (23), A36R (62), A56R (68), B5R (25, 45) and F13L (40) open reading frames (ORFs) of VV. All of these proteins have been demonstrated to play important roles in the formation, release and/or infectivity of EEV with the exception of the A56R gene, which encodes the viral hemagglutinin. None of these proteins affects the formation of IMV. Preliminary studies suggest that the IMV particle also contains proteins not found on the multiply enveloped forms of the virus. The ATI protein and the 4c protein are unique to IMV and although their biological relevance is not known they may represent an evolutionary relic. While VV does not occlude virions in ATIs, the closely related cowpox virus does (63). In that system, it has been demonstrated that the 4c protein is required for occlusion of virions in ATIs. It may be that the

association of the ATI and 4c proteins with the VV IMV particle is an abortive attempt at ATI formation.

The virus and its life cycle are complex and not completely understood. Throughout its life cycle the virus uses numerous cellular protein processing pathways to include proteolytic processing, phosphorylation, sulfation, glycosylation, and ADP-ribosylation (103). Here we review the acylation of VV proteins and discuss the significance of these proteins and their respective acyl modifications in the biology of VV.

1.1.2 Overview of Protein Acylation

Two classes of fatty acylated proteins exist in eukaryotic cells (53) and by extension are present in VV-infected cells as well. Labeling cultured cells with [^{3}H]-myristic acid ([^{3}H]-MA) or [^{3}H]-palmitic acid ([^{3}H]-PA), identifies distinct subsets of proteins. The distinction between the two classes can be determined in two ways. First, myristylation of proteins is a co-translational event and is inhibited by the addition of reagents which block translation. Under these conditions, palmitylation of previously translated proteins occurs normally while myristylation does not. Second, the palmitate-protein bond is labile in the presence of mild alkali or neutral hydroxylamine due to the thioester linkage while the amide-linked myristate is stable under the same conditions. Many palmitylproteins are membrane associated either directly through the palmitate moiety or as transmembrane proteins anchored by the fatty acid, although a few are secreted from cells. Cell-associated palmitylproteins are distributed throughout the cell, with the greatest concentration at the cell surface. Myristylproteins, on the other hand, may be cytoplasmic or membrane-associated. Some proteins are modified by both myristic and palmitic acids, with both acyl moieties contributing to protein function and localization.

Myristylation of proteins involves the transfer of myristate from myristyl-Coenzyme A to the amino-terminal motif MG*XXX*(S/T/A/C/N) (using the single-letter amino acid code) of proteins by the enzyme N-myristoyl transferase (NMT) (100) This motif bestows substrate specificity for the *Saccharomyces cerevisiae*-encoded enzyme, and although the human homologue has similar specificity, it may not be entirely the same. The initiating methionine is removed by methionine aminopeptidase during translation and NMT recognizes the newly generated amino-terminal glycine of the nascent peptide after approximately twenty residues are free of the ribosome. NMT transfers myristate to the glycine residue, after which the enzyme releases the peptide and translation continues normally. Mutations that change any of the conserved residues of the motif inhibit myristylation, with the greatest inhibition achieved by replacing the penultimate glycine with any residue (101). The residues at position six of the motif are less important, with low levels of myristylation occurring even if they are changed. It should be noted, though, that not all proteins containing the amino-terminal motif are myristylated, indicating that there are additional requirements. See Figure 1.2 for the "ball and stick" model of a multiply fatty acylated peptide.

Recently, myristylation has been demonstrated to occur on internal residues of proteins (95). Although the motif directing this modification has not been discovered, the myristate acceptor residues appear to be arginine or lysine (Figure 1.2). This may be due to the presence of free amines in their side-chains and is supported by experiments demonstrating their insensitivity to neutral hydroxylamine, suggesting amide linkage for this modification as well. The enzyme(s) responsible for internal myristylation is (are) unknown.

Palmitylation of proteins remains an enigma for researchers. Palmitylproteins are more aptly described as ester-type fatty acylated (86) proteins or even S-acylated proteins (73),

in reference to the sulfur atom in the side-chain of the acceptor cysteine. Some proteins are preferably modified by stearic or oleic acids over palmitic acid, but for the most part palmitate is the predominant fatty acid on these proteins with small percentages of them being modified by stearate and oleate or even arachidonate (87). While cysteine is the most common acceptor residue, serine or threonine may also serve as palmitate acceptors (8), so even the description of these proteins as S-acylated is not totally accurate.

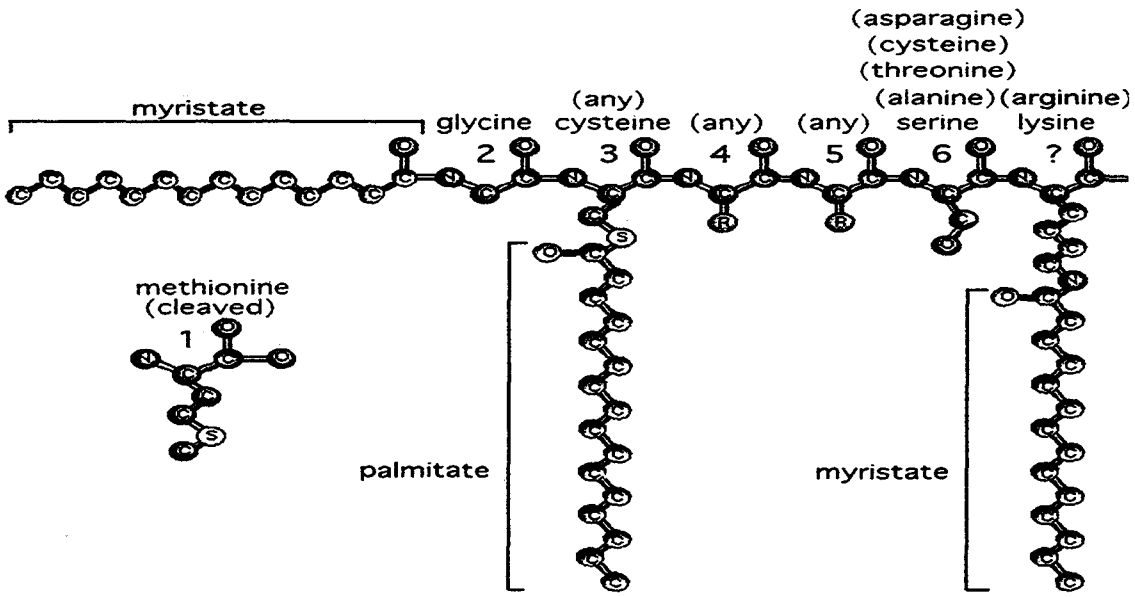

Figure 1.2. Structure of a hypothetical acylated peptide. A peptide consisting of the canonical amino-terminal myristylation motif is shown. The amino-terminal methionine is cleaved and the penultimate glycine residue is modified by the amide-bound 14-carbon fatty acid myristate. Cysteine in position 3 is modified by the thioester-linked 16-carbon fatty acid palmitate as in Type 4 palmitylproteins (see text). An internal lysine residue is modified by myristate as well. Alternate acceptable amino acids for each position are indicated in parentheses above the peptide backbone.

To date, a consensus motif directing palmitylation of proteins as well as the enzyme(s) responsible for the modification is (are) unknown. Palmitylprotein acyltransferase has been described as a membrane-bound component of cells, with *in vitro* activity detected in endoplasmic reticulum, Golgi and plasma membrane fractions of cells (4). The enzyme has a requirement for the activated form of palmitate, palmityl-coenzyme A, but little else is known about it. An enzyme capable of removing palmitate from proteins, palmityl-protein thioesterase, has been discovered (14, 22, 106), and is defective in infantile neuronal ceroid lipofuscinosis.

Some general characteristics of palmitylproteins are known and have allowed us to classify them into four subclasses (74). Type I palmitylproteins are transmembrane proteins that are modified on cysteines at or near the cytoplasmic membrane face. This group is typified by the G-protein coupled receptors and includes the vesicular stomatitis virus G and the influenza virus hemagglutinin proteins. The palmitylation of Type II proteins occurs in the carboxy-terminal region and is dependent on prior prenylation of cysteine in the C*AAX* motif at the extreme carboxy-terminus. Members of this group include the ras proteins. Types III and IV are dually fatty acylated in the amino-terminal region. Both groups are myristylated on glycine of the motif MG*XXX*(S/T/A/C/N). Type III palmitylproteins are modified on one or more cysteines within the first 10 to 20 amino acids while Type IV palmitylproteins are modified on cysteine immediately following the myristylated glycine residue (Figure 1.2). Efficient palmitylation of Types III and IV is dependent on prior myristylation. The alpha subunits of the heterotrimeric G-proteins are grouped as Type III or IV.

1.2 Identification of VV Acylproteins

Since the discovery that VV encoded a palmitylated protein localized to the TGN in infected cells and present on the surface of virions (38), a great deal of work has been performed to discover other VV acylproteins, their role in the VV life cycle and the significance of the modification. Workers in our laboratory have confirmed the existence of at least 6 myristylproteins (27) and 8 palmitylproteins (16) encoded by VV. Their salient features are summarized in the Vaccinia Acylprotein table.

1.2.1 Identification of VV Myristylproteins

The discovery of the myristylation motif allowed workers in our lab to undertake a systematic approach to identify VV-encoded myristylproteins. The entire sequence of the Copenhagen strain of VV is known (32), and by deduction, the amino acid sequences of the proteins it encodes. Four of the potential peptide sequences contain the amino-terminal motif MG*XXX*(S/T/A/C/N). They are encoded by the A16L, E7R, G9R and L1R ORFs, producing proteins with predicted masses of 43.6, 19.5, 38.8 and 25 kDa respectively. Labeling VV-infected cells with [^{3}H]-MA and resolution by SDS-PAGE demonstrates that six polypeptides incorporate the label with electrophoretic mobilities indicating masses of 92, 39, 36, 25, 17, and 14 kDa. By *in vitro* transcription and translation, the identities of the 39-, 36-, 25-, and 17-kDa proteins were demonstrated to be encoded by the A16L, G9R, L1R and E7R ORFs respectively, as predicted (29, 57). In this system, the ORFs were cloned into plasmid vectors downstream of T7 promoters. By transcription using T7 RNA polymerase and translation in the presence of [^{3}H]-MA using wheat germ extracts the proteins were demonstrated to incorporate the label. Mutants of these proteins, in which the penultimate glycine was changed

Vaccinia Acylproteins.

Protein/Gene	Modification	Site of Modification	Localization	Ref.
15K/A14L	myristate	unknown	ERGIC[c], IMV[d] envelope	(77)
unnamed[a]/A16L	myristate	MG*XXX(S/T/A/C/N)[b]	cytosol	(57)
ATI protein/A25L	myristate palmitate	unknown unknown	cytosol, IMV	(56)
unnamed/A33R	palmitate	unknown	TGN[e], IEV[f], CEV[g], EEV[h] outer envelope	(81)
gp42/B5R	palmitate	unknown	TGN, IEV, CEV, EEV outer envelope, plasma membrane	(25, 45)
unnamed/E7R	myristate	MG*XXX(S/T/A/C/N)	cytosol	(57)
p37/F13L	palmitate	cysteine 185 cysteine 186	TGN, IEV, CEV, EEV outer envelope	(40)
unnamed/G9R	myristate	MG*XXX(S/T/A/C/N)	unknown	(57)
M25/L1R	myristate	MG*XXX(S/T/A/C/N)	IMV envelope	(71)
p14/unknown	palmitate	unknown	virion associated	(16)
p17/unknown	palmitate	unknown	virion associated	(16)

[a] Unnamed VV proteins are referred to by open reading frame in the text., [b] amino-terminal myristylation motif. * indicates the myristyl acceptor glycine residue after cleavage of the initiating methionine., [c] endoplasmic reticulum-Golgi intermediate compartment, [d] intracellular mature virus, [e] *trans*-Golgi network, [f] intracellular enveloped virus, [g] cell-associated enveloped virus, [h] extracellular enveloped virus

to alanine, did not incorporate label in this system. Antibodies directed against these proteins immunoprecipitated [^{3}H]-MA labeled proteins from infected cells that co-migrated with the *in vitro* translated proteins as well as proteins from whole cell extracts, confirming their modification by myristate *in vivo* as well. High-performance liquid chromatography (HPLC) of fatty acids extracted from these proteins demonstrated that myristate was the predominant modifying moiety but palmitate was also present. This is most likely the result of interconversion of the myristate post-modification, as the addition of [^{3}H]-PA to infected cells did not label these same proteins efficiently.

The identity of the 92-kDa myristylprotein was deduced by Martin *et al* (56). Although a number of VV-encoded proteins are known to migrate with similar electrophoretic mobility, only one matched the observed expression kinetics. Pulse-chase labeling of infected cells with [^{3}H]-MA demonstrated that the label was incorporated most efficiently at very late times post-infection. The only protein known to be expressed by VV in this manner was the ATI protein. To confirm that the ATI protein was indeed myristylated, cells were infected with the Western Reserve (WR) or Copenhagen (COP) strains of VV or the closely related cowpox virus and cultured in the presence of [^{3}H]-MA. Cowpox is known to encode the full-length (160 kDa) ATI protein while WR encodes a truncated 92 kDa form. COP does not encode the ATI protein due to a frameshift mutation that introduces a stop codon near the initiating methionine. Resolution of the labeled, infected cell extracts by SDS-PAGE demonstrated that COP did not encode a high molecular weight myristylprotein while cowpox and WR encoded 160-kDa and 92-kDa myristylproteins respectively. Confirmation of these findings was obtained by the development of antiserum to the ATI protein that was used to immunoprecipitate the [^{3}H]-MA labeled protein from infected cell extracts.

The ATI protein does not contain the amino-terminal myristylation consensus motif, suggesting that the modification occurred either at an internal site or that the label was incorporated as result of interconversion of myristate to palmitate. It turns out that both scenarios are probably correct. HPLC analysis of fatty acids extracted from the ATI protein confirmed that myristate and palmitate were both present on the protein, a finding that is not uncommon for myristylproteins. It was also found that translational inhibitors reduced the efficiency of label incorporation but did not block it entirely, suggesting that the protein was palmitylated, while at the same time, treatment with neutral hydroxylamine did not entirely remove the label, suggesting stable amide linkage for the fatty acid. Addition of [^{3}H]-PA to infected cell cultures also resulted in label incorporation by the ATI protein. Evidence presented in Chapter 2 is contradictory to these findings though. It may be that the 92-kDa palmitylprotein is in fact a distinct but co-migrating protein.

No VV-encoded protein is predicted to match the size of the 14-kDa myristylprotein and contain the amino-terminal myristylation motif, suggesting that this protein represents the second example of an internally myristylated VV protein. In a report by Rodriguez *et al* (77) this protein was identified as being encoded by the A14L ORF. In arriving at this conclusion, the authors labeled infected cells with [^{3}H]-MA and immunoprecipitated the A14L protein (which migrated as a 15-kDa protein in their system), demonstrating that it incorporated label. They did not, however, demonstrate that the label was incorporated as myristate and not some other long chain fatty acid arising from interconversion. In addition, they suggested a number of internal glycine residues as possible sites of modification. This is not likely to be the case as glycines do not have free amino groups to react with the carboxyl moiety of the fatty acid. It is more likely that the protein is modified by myristate on lysine(s) or arginine(s) (which have free amines) at

internal sites or that the fatty acid was converted to palmitate or other long-chain fatty acid and added through an ester-type linkage to cysteine, serine or threonine. Further analysis of this protein needs to be performed to confirm that it is truly myristylated and to define the exact nature of the acyl modification.

1.2.2 Identification of VV Palmitylproteins

The first report of a VV-encoded fatty acylprotein was by Hiller *et al* (38). They described a 37-kDa palmitylated protein (p37) present in Golgi membrane fractions of infected cells and in the outer envelope of EEV. Since this initial finding the protein has been confirmed to be the product of the F13L ORF in numerous reports (9, 40, 65). Additionally, Child and Hruby have demonstrated the existence of at least five more palmitylproteins induced by VV (16). By addition of [^{3}H]-PA to infected cells and SDS-PAGE resolution of the labeled proteins, they confirmed that VV encodes proteins that incorporate the label with electrophoretic mobilities of 92, 42, 37, 26, 17, and 14 kDa. To date, no laboratory has undertaken a directed effort to identify these proteins although the identities of some have been obtained as an aside in other efforts to characterize VV proteins.

Isaacs *et al* (45) confirmed that addition of [^{3}H]-PA to VV-infected cells resulted in the specific labeling of a 42-kDa glycoprotein (gp42). Antibodies known to react with the product of the B5R ORF immunoprecipitated gp42, confirming its identity. It most likely is a member of the Type 1 palmitylprotein subclass. Following cleavage of the signal sequence the protein is oriented in the membrane as a type I transmembrane protein, spanning it only once, and having a very short carboxy-terminal tail exposed to the cytoplasm. The protein transits the cell using the normal protein export pathway, acquiring glycosyl moieties in the

process, and eventually resides in the TGN and plasma membranes. The palmitate modification site is unknown although it is likely that it is in the carboxy-terminal cytoplasmic tail, that contains a number of cysteine residues.

The 26-kDa palmitylprotein that we identified actually migrates as four distinct species in reducing SDS-PAGE gels; as a 55 kDa species and as 3 species of 23 to 28 kDa. Glycosylation inhibitors block the appearance of the three slower migrating species, suggesting a complex glycosylation pattern. This protein was once thought to be the product of the A34R ORF (84) but has since been demonstrated to be encoded by the A33R ORF (81). The misidentification is an understandable one. When the protein was first identified as the product of the A34R ORF, it was known that the A34R protein (gp22) was a glycoprotein migrating in SDS-PAGE gels with electrophoretic mobilities of 22 to 24 kDa and was unique to the outer envelope of EEV (23). By using a monoclonal antibody generated to EEV-specific proteins, a palmitylprotein exhibiting similar characteristics was immunoprecipitated from [^{3}H]-PA-labeled infected cell extracts. It has since been determined that the monoclonal antibody used is specific for the product of the A33R ORF. The A33R protein (referred to as gp23-28) is predicted to be a type II transmembrane protein with a short amino-terminal cytoplasmic tail. Based on this prediction, the A33R protein is most likely a member of the Type I palmitylprotein subclass.

Since the initial discovery that p37 was encoded by the F13L ORF, we have made a systematic effort to identify the site of modification and understand the significance of the acyl moiety in the biology of VV. Although the primary amino acid sequence necessary for recognition by palmitylprotein acyltransferase and subsequent transfer of palmitate to proteins is not known, Ponimaskin and Schmidt have identified sequence constraints for numerous viral glycosylated palmitylproteins (69). Their observations indicate that transmembrane proteins containing

cysteine residues from within six amino acids into the transmembrane region (on the cytoplasmic side) to ten amino acids away from the membrane:cytoplasm boundary are efficient substrates for palmitylation. Cysteines located in the middle of the membrane, on the extracellular face, or more than 10 amino acids away from the membrane:cytoplasm boundary, are not efficiently palmitylated. Cysteines alone, near the membrane, are not sufficient to direct palmitylation. Insertion of cysteine residues at the membrane boundary of nonpalmitylated proteins did not convert them to substrates for palmitylation.

It was once thought that p37 was a transmembrane protein, as it is known to be membrane-associated within infected cells, fractionates with membrane-bound components of cells and is associated with the EEV envelope. In fact, the membrane association is peripheral in nature and interestingly is mediated by the palmityl moiety alone (34, 91). Treatment of p37-containing membrane fractions of cells with neutral hydroxylamine results in the release of p37 from the membrane, leaving it free-floating in the aqueous fraction. Type III and IV palmitylproteins are peripherally associated with membranes but are also substrates for myristylation. p37 is not myristylated so it cannot be classified as Type III or IV. It may be appropriate to suggest that p37 is a member of a novel subclass of palmitylproteins - one in which palmitylation is necessary and sufficient for peripheral membrane association.

In our analysis of the primary amino acid sequence of p37, using the guidelines of Ponimaskin and Schmidt, we were unable to predict potential modification sites in the protein. Therefore, we analyzed, by sequence alignment, numerous palmitylproteins whose sites of modification were known, and we discovered a loosely conserved motif. We found that cysteine (or a cysteine doublet) within a transmembrane or hydrophobic stretch of amino acids that was preceded by two aliphatic residues and followed by another served as a substrate for palmitylation. Comparison of

this motif to the p37 sequence yielded one possible site for modification - a cysteine doublet in the middle of a hydrophobic domain in the central region of the protein. Mutation of one or the other of these cysteines to serine reduced the efficiency of palmitylation while mutation of both to serine inhibited it completely (34).

At least three VV-encoded palmitylproteins are unidentified. The 14-, 17-, and 92-kDa proteins, observed by SDS-PAGE resolution of labeled, infected cell extracts, are encoded by unknown ORFs. In addition to these easily detected proteins, there may be others not so readily observed.

1.3 Biological Significance of VV Acylproteins

1.3.1 Myristylproteins

The L1R protein (also referred to as M25) is the best characterized VV myristylprotein regarding protein function and significance of the myristyl modification. The protein is localized to the envelope of the IMV particle and is predicted to span the membrane twice (71). There, it presumably acts as a necessary structural component of the developing virion (see Figure 1.3 for hypothetical membrane topologies and virion association of all VV acylproteins). A mutant virus in which transcription of the L1R ORF was inhibited by the *lac* repressor unless isopropyl-thio-galactopyranoside (IPTG) was added to the medium, could not progress past the virion morphogenetic stage at which the core condenses to form IMV (72). By electron microscopy, numerous immature virions were observed as membrane-enclosed viroplasm. Analysis of the virion core proteins confirmed that proteolytic processing to their mature forms did not occur either,

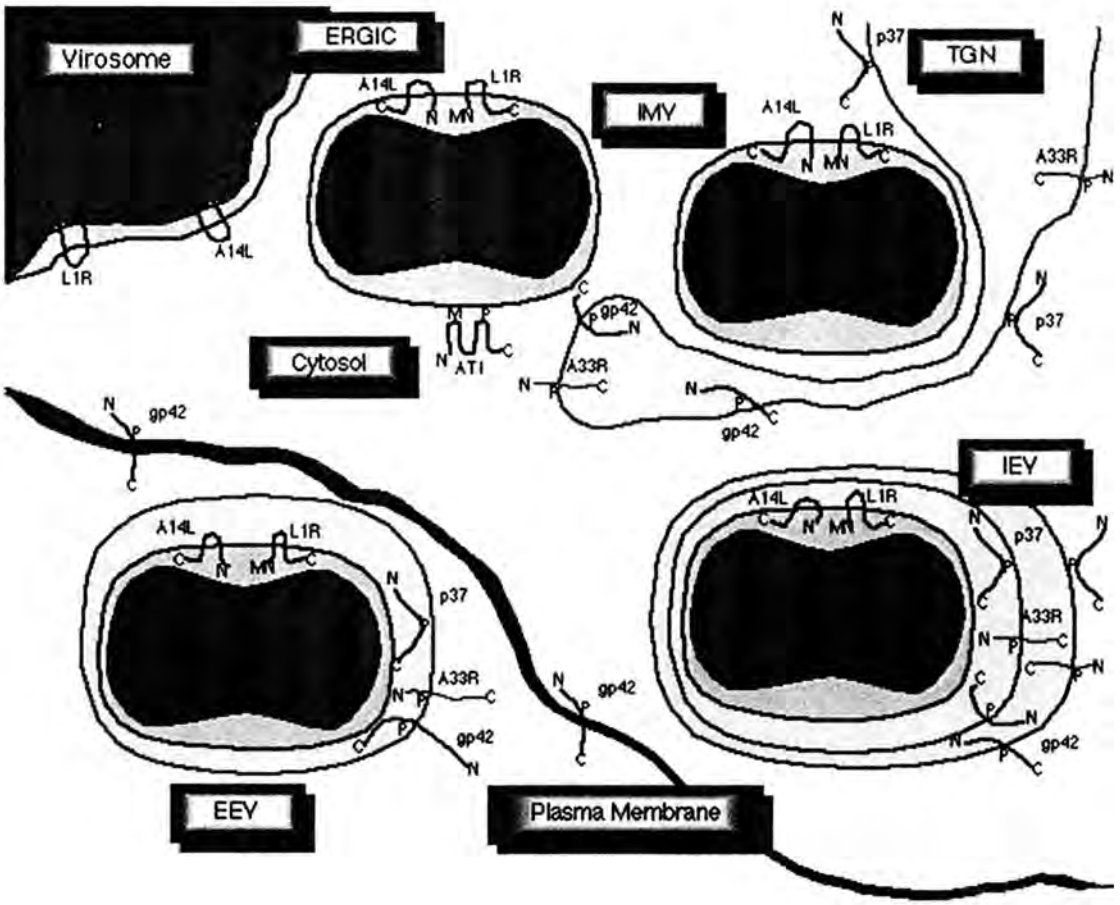

Figure 1.3. Cellular and viral distribution of VV acylproteins. A close-up view of an infected cell is shown, highlighting membranes and structures involved in virion morphogenesis. Membrane crescents derived from the endoplasmic reticulum-Golgi intermediate compartment (ERGIC) form in the vicinity of the virosome leading to the production of intracellular mature virus (IMV). IMV is enveloped by membranes from the *trans*-Golgi network (TGN) forming intracellular enveloped virus (IEV). Following fusion of the outermost IEV envelope with the plasma membrane, extracellular enveloped virus (EEV) is released into the extracellular environment. Hypothetical protein:membrane interactions are shown. The amino- and carboxy-terminal ends of the proteins are indicated by "N" and "C" respectively. Myristyl and palmityl modifications are indicated by "M" and "P" respectively.

demonstrating the co-dependency of both processes. This virus could be rescued by addition of IPTG or by transient expression of the wild-type L1R from a transfected plasmid carrying the gene. Transfection with a plasmid encoding a mutant L1R, in which the myristyl acceptor glycine had been changed to alanine, could not rescue the virus. It is clear from these findings that L1R plays a significant role in the maturation of virions. Additionally, the myristylation of L1R appears to be essential for protein function. Although the myristylation of L1R may assist in the correct folding of the protein, it is also involved in targeting the protein to virion membranes. A fusion protein, in which the first twelve amino acids from L1R were fused to the entire bacterial chloramphenicol acetyl transferase gene, was myristylated and present in virions (70). The molecular mechanisms by which L1R functions are unknown.

Although the ATI protein is known to function as a structural matrix for the formation of ATI bodies in other poxviruses (63), it has no demonstrable function in VV (1). As stated above, VV expresses a truncated form of the protein that is conserved across numerous strains with the exception of the COP strain, which does not express the ATI protein. There has been some speculation that it may serve as an immune decoy or deterrent to phagocytosis but there are no supporting data for this hypothesis (19). Our analysis of the significance of the acyl modifications has been hindered by the lack of an observable phenotype attributable to this protein. Likewise, the large size of the protein has made mutational analysis of the protein a lengthy task.

The 15-kDa myristylprotein product of the A14L ORF has been demonstrated to be associated with membranes of the endoplasmic reticulum-Golgi intermediate compartment (77). In addition to acylation, the protein is phosphorylated. The functions of both modifications is unknown. The protein has two hydrophobic domains predicted to be alpha-helical in structure, characteristic

of transmembrane proteins. The protein exists as part of a complex with the A17L (21 kDa) and the A27L (14 kDa) proteins with unknown stoichiometry (77). These proteins collectively are involved in the formation of previrion membrane crescents at the site of virion development and are found associated with the outer envelope of immature virions and IMV. They presumably comprise a component of the inner membranes of IEV, CEV and EEV. It has been recently reported that both the A14L and A17L gene products are necessary for the recruitment of intermediate compartment membranes to virus factories (78).

The functions of these three remaining myristylproteins have not yet been determined. Hydropathy and secondary structural predictions suggest that the A16L and G9R proteins might be targeted to membranes while the E7R protein is largely hydrophilic (57). Surprisingly, subcellular fractionation of infected cells into soluble and particulate fractions demonstrated that the A16L and E7R proteins were soluble. The G9R protein was not detected in this assay, suggesting that it is made in low quantity, or that it is labile and was degraded in the purification process. Neither of these three proteins was detected in purified virions.

1.3.2 Palmitylproteins

The three VV palmitylproteins that have been identified all prove essential to the formation of IEV, virion egress and release of EEV/CEV from cells. This process has been the subject of intense scrutiny by numerous laboratories, all providing pieces to the puzzle. The IMV particle, while fully infectious and representing the majority of virus purified from tissue culture systems, most likely is an intermediate virion *in vivo*. IMV are targeted to the TGN and by budding through it acquire additional membranes, forming IEV (38). The formation of IEV is dependent

on expression of the A27L gene encoding the 14-kDa protein found on the surface of IMV (75), as well as the products of the A33R (gp23-28) (80), A36R (gp45-50) (62), B5R (gp42) (26, 45) and F13L (p37) (9) genes, all specific for the multiply enveloped forms of virions. By a poorly understood mechanism, IEV are able to recruit actin, forming thick filaments that propel the virus unidirectionally to the cell surface, sometimes into neighboring uninfected cells (17). An additional requirement for this task is the A34R gene product, gp22 (110), which is also a component of IEV, CEV and EEV outer membranes. Deleting or inhibiting the expression of any of these genes results in the accumulation of IMV and/or inhibition of virus dissemination. The exact role each of these proteins plays is not clear.

Recent data suggest that p37 is a member of the phospholipase D superfamily based on conserved sequences (15, 97). Although no phospholipase D activity could be detected in VV-infected cell extracts, the purified protein was demonstrated to possess phospholipase A and C activity (2). Mutation of the sequences in p37 that are conserved in the phospholipase family of proteins resulted in a protein that was no longer capable of functioning regarding IMV envelopment, suggesting that phospholipase activity is a necessary protein function. Palmitylation of p37 is also necessary for function (34). In this thesis we have demonstrated that p37 is palmitylated on both cysteines of a doublet in the central region of the protein. A mutant virus expressing a nonpalmitylated form of p37 was defective for envelopment of IMV and subsequent release of virus from the cell. Wild-type p37 is normally a component of TGN membranes but the nonpalmitylated p37 was soluble in the cytoplasm of infected cells. It would appear that VV requires the very specifically localized phospholipase activity of p37 to progress past the IMV morphogenetic stage. The substrates of this enzyme are unknown.

The 42-kDa glycosylated palmitylprotein, gp42, is a type 1 transmembrane protein present on the surface of infected cells and on the outer envelopes of IEV, CEV and EEV (65). A large amino-terminal region of the protein is exposed to the extracellular environment and contains 4 short consensus repeats characteristic of complement control factors. VV encodes a secreted homologue of gp42 that has been demonstrated to bind complement (44), but whether gp42 functions in an analogous manner is not known. Deletion mutants of the B5R ORF demonstrate the significance of this protein in the VV life cycle. Viruses that do not make gp42 are attenuated *in vivo* and form small plaques in tissue culture (109). This is a direct result of the inability of these viruses to produce normal amounts of EEV and not related to the loss of the extracellular short consensus repeats. Restoration of the transmembrane domain and short carboxy-terminal cytoplasmic tail rescues B5R deletion viruses, suggesting that the extracellular domain is dispensable for the formation of EEV (49). Not only is the carboxy-terminal region all that is necessary for protein function, it is also sufficient to target heterologous fusion proteins to EEV particles (49). This does not preclude the possibility of a role for the extracellular domain though. Based on our predictions, the carboxy-terminus of gp42 is also the site of palmitate modification. The significance of the modification is unknown, but considering the importance of this region it will be of interest to investigate it.

The A33R protein, also referred to as gp23-28, is targeted to the membranes of the TGN and is predicted to be oriented as a type II transmembrane protein with the amino-terminal region exposed to the cytoplasm (81). After envelopment of IMV by TGN membranes, the protein is present in the outer envelopes of IEV, CEV and EEV and is required for their formation (80). Deletion of the A33R gene results in a small-plaque phenotype, indicating a reduced dissemination efficiency. The exact role this protein plays is unknown, as is the site of palmitate modification.

1.4 Discussion

VV is a large virus with a complex life cycle. This complexity is due to more than 200 gene products expressed in VV-infected cells. To maintain regulatory control and correct targeting, the virus has adopted many cellular protein processing pathways for its own use. This in itself highlights the advantages of using VV as a transient mammalian expression system. Proteins expressed in VV-infected cells are modified in their native manner and are targeted intracellularly or transit the cell normally. Protein modifications known to occur in VV-infected cells include proteolytic processing, phosphorylation, sulfation, glycosylation, ADP-ribosylation and as reviewed here, acylation (103). The acylation of VV polypeptides in many ways models cellular systems, having similar motifs specifying the modifications, and playing analogous roles in protein function and in targeting. Although we have learned much from these proteins, there is still much work to be performed in completing their characterization.

Through our efforts to characterize the L1R protein we have demonstrated its essentiality to the virus as well as the significance of the myristyl modification to protein function. Our focus has now shifted toward gaining a better understanding of the protein:membrane interaction. With a knowledge of the membrane topology of L1R it may be possible to dissect functional domains of the protein and gain insight into the molecular mechanism of action. Additionally, it is not known that L1R has any protein partners, and it will be of interest to determine if there are any.

The roles that E7R, G9R and A16L play in the VV life cycle are unknown. Current efforts involve the construction of recombinant viruses in which these genes are under inducible control. This should allow us to ascertain the necessity of these

proteins to the virus. If significant, we would then further the research by an analysis of the biological role the myristyl moieties play.

Much is already known of p37 and the significance of its palmitylation (see Chapter 3) but, based on recent reports of phospholipase activity, it will be of interest to discover the *in vivo* substrates for this enzyme. Considering the numerous protein:membrane interactions necessary for envelopment of IMV to form EEV, one can only speculate on possible roles that a phospholipase would play in this process.

Both gp42 (B5R) and the gp23-28 (A33R) are essential for dissemination of enveloped virions, but why? No enzymatic activity has been proposed for either of these proteins, and although gp42 has homology to complement control proteins, that entire domain is dispensable for the formation of enveloped virus. The gp23-28 protein does not share homology with any protein other than its homologues in other poxviruses so no hypothesis regarding function can be made. While many laboratories are working to determine the function of these proteins, we are investigating the importance of the palmitate modification. Unlike p37, which requires palmitylation for membrane association, these proteins are inserted into the membrane as a function of their primary amino acid sequence. In other systems, the palmitylation of transmembrane proteins has been demonstrated to be dispensable for membrane association and in some cases, function (48), while in others it is necessary for both (112).

The goals of this thesis are to identify the unknown VV-encoded palmitylproteins and determine their sites of modification. The major VV-encoded palmitylprotein, p37, will then be analyzed further for the significance of its palmitate modification.

Chapter 2

Identification and Analysis of Vaccinia Virus Palmitylproteins

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2.1 Introduction

Palmitylation of proteins involves the transfer of palmitate, a 16-carbon saturated fatty acid, from palmityl-coenzyme A to cysteines, serines or threonines through thioester or ester linkage (55, 83, 85). The modification is made post-translationally and in most cases by an unidentified enzyme or enzymes referred to as palmitylprotein acyltransferases (PATs) (4, 88). Some reports suggest that at least for a small number of proteins a nonenzymatic or autocatalytic mechanism is sufficient to mediate acyl transfer (3, 5, 7). The modification is reversible *in vivo* and often modulates function or subcellular localization of proteins.

Although many cellular and viral palmitylproteins have been characterized regarding site(s) of modification, a definitive structural motif specifying palmitylation of proteins has not been identified. Palmitylproteins have been separated into four separate classes based on membrane topology, prior modification by myristate or isoprenyl moieties, and relative to the termini, which region of the protein is modified (74). We have previously reported that the palmitate acceptor residue(s) of proteins may be predicted by the consensus motif, Hydro*1-12*AACA* (35). The hydrophobic element (Hydro*) may be a transmembrane domain, a hydrophobic sequence of amino acids or a glycine residue that has been co-translationally myristylated. It is followed by a 1- to 12-amino acid spacer that precedes aliphatic residues (*A*) surrounding the palmitylated cysteine (C). This motif is based on work in our laboratory as well as the work of others (6, 46, 69, 86, 90, 105).

The life cycle of VV is well characterized (61) (see Figure 1.1). Upon entry into cells the virus expresses a distinct subset of early genes leading to uncoating and release of its genome. Genome replication begins followed by intermediate gene expression. The intermediate gene products primarily serve as

transcription factors for the late genes. Following late gene expression, virion assembly rapidly ensues in concentrated cytoplasmic foci of viral DNA, proteins and lipids, termed viroplasm. The formation of the first virion membrane is controversial in that it is not entirely clear whether it is derived from the intermediate compartment of the host (93) or produced by virus-specific membrane forming machinery (41). Nevertheless, membrane crescents encircle viroplasm containing a single copy of the genome and proteinaceous components of the core. As the core condenses to the biconcave shape typical of poxviruses, the first infectious virions are produced, termed IMV. Depending on the strain of virus or cell system used, a fraction of IMV ranging from 25-40% is targeted to the TGN where, by budding through the compartment, two additional membranes are acquired, forming IEV (66). IEV may exit the cell by two mechanisms. The first is a passive release that is not well characterized. The second mechanism involves the formation of "actin rockets" that propel the virus unidirectionally through and out of the cell, sometimes directly into neighboring uninfected cells (17). Outside of the cell, virions may remain attached (or reattach) to the plasma membrane or be released into the extracellular environment. Virions attached to the cell are referred to as CEV, while those free-floating in the extracellular environment are referred to as EEV.

VV serves as an ideal model system for the study of protein processing for several reasons: VV proteins (or exogenous proteins expressed either transiently from transfected plasmids or from recombinant VV) are subject to modifications typical of mammalian systems, to include sulfation (65), phosphorylation, ADP-ribosylation, glycosylation and, as highlighted here, acylation (103). Its genes are expressed in the cytoplasm without mRNA processing, allowing conceptual translation directly from genomic sequence. We have previously observed that many VV proteins are acylated providing numerous subjects for study

In this chapter we report on the identification of previously unknown palmitylproteins encoded by the A22R, A36R and A56R (COP strain) ORFs of VV. Then, by a biochemical characterization of the proteins and comparison to the palmitylation motif, Hydro*1-12*AACA*, we were able to accurately predict the modification sites for gp45-50 (A36R), gp86 (A56R), gp42 (B5R) and p37 (F13L).

2.2. Materials and Methods

2.2.1 Cells and Viruses

BSC_{40} (African green monkey kidney) and RK13 (rabbit kidney) cell lines were cultured in Eagle's minimal essential medium supplemented with 10% (v/v) fetal calf serum, 2 mM L-glutamine, 10 µg/ml gentamycin-sulfate (MEM-10 LG/GS) at 37°C in a 5% CO_2 humidified atmosphere. Upon infection with VV, the cells were then cultured in Eagle's minimal essential medium supplemented with 2 mM L-glutamine, 10 µg/ml gentamycin-sulfate (MEM LG/GS) at 37°C in a 5% CO_2 humidified atmosphere. The COP, IHD-J, and WR strains of VV were routinely propagated and titered in BSC_{40} cells as previously described (24). The recombinant VV, vTF7-3 (30), was used for transient expression of genes cloned into the plasmid vector, pTM1:6xHis (described below). This virus contains of copy of T7 *gene 1* inserted at the thymidine kinase locus of the VV genome and expresses T7 RNA polymerase nearly constitutively during infection. Genes downstream of a T7 promoter are expressed to high levels when present in the cytoplasm of vTF7-3-infected cells. Standard methods were used to propagate and titer this virus as well.

2.2.2 Metabolic Labeling of VV Proteins with [^{3}H]-Myristic Acid and [^{3}H]-Palmitic Acid

Labeling of COP, IHD-J and WR acylproteins: [9,10 ^{3}H(N)]-Myristic acid (50 Ci/mM; [^{3}H]-MA) and [9, 10 ^{3}H(N)]-palmitic acid (50 Ci/mM; [^{3}H]-PA), purchased as ethanolic solutions, were dried by nitrogen overflow. The lipids were then resuspended in dimethyl sulfoxide (DMSO) at a concentration of 10 μCi/μl. BSC_{40} cells were grown to confluency in 35 mM wells (10^6 cells/well). The cells were synchronously infected with either COP, IHD-J or WR strains of VV at a multiplicity of infection (moi) of 10. At 4 hours post-infection (hpi), [^{3}H]-MA or [^{3}H]-PA was added to a final concentration of 100 μCi/ml in no more than 10 μl of DMSO. At 24 hpi the cells were harvested and resuspended in reducing sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer and frozen until needed.

Time course labeling: BSC_{40} cells were grown to confluency in 35 mM wells. The cells were synchronously co-infected with COP and WR strains of VV at a moi of 10 each (final moi = 20). Labeled lipids were added to a final concentration of 100 μCi/ml in no more than 10 μl of DMSO. The labeling periods included the 0-1, 1-2, 2-4, 4-8, and 8-24 hpi time-points. Infected cells were immediately harvested at the end of the labeling periods and resuspended in reducing SDS-PAGE sample buffer and frozen until needed.

Labeling infected cells for subcellular fractionation and purification of IMV and EEV: BSC_{40} cells were grown to confluency on 150 mM tissue culture plates ($2x10^7$ cells). The cells were co-infected with the COP and WR strains of VV at a moi of 10 each (final moi = 20). At 4 hpi [^{3}H]-PA was added to a final concentration of 100 μCi/ml. At 24 hpi both the culture medium and the infected cells were collected for purification of IMV and EEV and for subcellular fractionation of VV

palmitylproteins. The culture supernatant was clarified of cellular debris by centrifugation at 700 x *g* for 20 minutes. Labeled EEV was then pelleted from the clarified culture medium by centifugation at 100,000 x *g* for 30 minutes. The EEV pellet was resuspended in 500 µl of phosphate-buffered saline (137 mM NaCl, 2.7 mM KCl, 4.3 mM $Na_2HPO_4 \cdot 7H_2O$, 1.4 mM KH_2PO_4; PBS), titered, and then frozen until needed. The infected cells were resuspended in 6 ml of PBS, rapidly frozen, then thawed to lyse cells. To ensure cell lysis the cellular extracts were vortexed vigorously while still partially frozen using the ice crystals to homogenize the cell extracts. One ml of the infected cell lysate was set aside for subcellular fractionation. IMV was purified from the remaining 5 ml of cell lysate by using CsCl gradients following techniques standard for VV (67).

2.2.3 Differential Centrifugal Subcellular Fractionation of VV-Infected Cell Extracts

From the 1-ml fraction of infected cell lysates (approximately $3x10^6$ cells), 100 µl was removed and set aside as the total cell extracts (TCE). The remaining 900 µl was centrifuged at 700 x *g* for 10 min to pellet nuclei. The pellet was resuspended in 900 µl of PBS and frozen until needed. The supernatant was centrifuged at 20,000 x *g* for 30 min to pellet cytosolic membranous components and whole virions. The pellet (P20) was resuspended in 900 µl of PBS then frozen until needed. The supernatant containing the soluble components of the cytoplasm (S20) was transferred to another microfuge tube and then frozen as well.

2.2.4 Sodium Dodecylsulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE), Affinity Blots and Fluorography of VV Proteins

For resolution of VV proteins by SDS-PAGE, all samples were resuspended in 1X reducing SDS-PAGE sample buffer. To minimize reduction of the thioester-bonded palmitate, samples were heated to 70°C for 1 min, then rapidly cooled to 4°C before loading the gels. All gels used were 12.5% acrylamide:bis-acrylamide (30:0.8). For affinity blots, SDS-PAGE gels were electroblotted to nitrocellulose by standard western blotting techniques (99) and then probed using Ni^{+}-activated horseradish peroxidase, which binds histidine-tagged proteins (see below). The blots were developed by using chemiluminescent reagents and exposure to film. To detect [^{3}H]-PA-or [^{3}H]-MA-labeled proteins in gels, the gels were first dehydrated by immersion in DMSO for 30 minutes. The gels were then impregnated with diphenyloxazole (PPO) by immersion in a 22.2% PPO:DMSO solution for 1 hour (13). The PPO was precipitated in the gel by immersion in water for 30 minutes. The gels were then dried and exposed to film.

2.2.5 Identification of Candidate Palmitylproteins

The identification of candidate VV-encoded palmitylproteins was made using a number of criteria: We have observed (see Results) that VV palmitylproteins are primarily "late" proteins, have relative molecular masses of 14, 17, 23-28 (a single protein), 37, 42, 46, 86, and 94 kDa, as determined by SDS-PAGE, and are specific for EEV. Focusing then, on VV gene products that matched these criteria, we then scanned the conceptually translated polypeptides from the COP strain of VV for sequences that fit with our previously reported palmitylation motif, Hydro*1-12*AACA*. "Hydro" may be a previously myristylated

glycine, a transmembrane domain, or a hydrophobic stretch of amino acids usually at least 10 residues in length. The hydrophobic element is usually followed by a 1- to 12-amino acid spacer then a cysteine (C) residue (or residues) surrounded by aliphatic amino acids (*A*).

2.2.6 Cloning and Transient Expression of Candidate Palmitylproteins

All the candidate palmitylprotein genes were cloned into pTM1:6xHis, constructed as follows: The L5R gene of VV was amplified from IHD-J genomic DNA by polymerase chain reaction (PCR) using primers L5R-5' (5'-CCGGAATTCATATGGAGAATGTTCCT AATG-3'), and L5R-3'6xHis (5'-CCAATGCATTGGTTCTGCAGTTTCAT CAGTGGTGGTGGTGGTGGTGGGATCCTCTGCGAAGAACATCG-3'). The PCR product was treated with restriction enzyme *Eco*RI, generating an overhang at the 5' end of the product and leaving the 3' end blunt, and then ligated into *Eco*RI/*Stu*I-restricted pTM1 producing the plasmid pTM1:L5R-6xHis. This placed the 5' end of the L5R gene downstream of the T7 promoter and encephalo-myocarditis virus 5'-untranslated region/internal ribosomal entry site (EMC-leader) on pTM1. The normal L5R stop codon was not included in the PCR primer and was instead replaced by sequences that, following transcription and translation, resulted in the addition of a glycine and a serine followed by six histidines at the carboxy terminus of the protein. The remaining candidate palmitylprotein genes were cloned into pTM1:L5R-6xHis. To do this, the L5R coding sequence was excised by restriction with *Nde*I and *Bam*HI, leaving the 6-histidine tag sequence intact on the plasmid. All other candidate palmitylprotein genes were PCR-amplified from IHD-J genomic DNA with the exception of the A56R gene, which was amplified from COP and WR genomic DNA as well as IHD-J. The A14L gene was amplified using primers A14L-5' (5'-CCGGAATTCATATGGACATGATGCTTATG-3') and A14L-3' (5'-

GCGGGATCCGTTCATGGAAATATCGCTATG-3'); the A22R gene was amplified using primers A22R-5' (5'-CCGGAATTCATATGTCGTCA CCGATG-3') and A22R-3' (5'-GCGGGATCCCATTTTTTTTATGTAATTC CTAG-3'); the A33R gene was amplified using primers A33R-5' (5'-CCGGAATTCATATGATGACACCAG-3') and A33R-3' (5'-GCGGGA TCCGTTCATTGTTTTAACAC-3'); the A34R gene was amplified using primers A34R-5' (5'-GCATCAGCCCCATATGAAATCGCTTAATAGACAA ACTGTAAG-3') and A34R-3' (5'-GCGGGATCCCTTGTAGAATTTTTTAA CAC-3'); the A36R gene was amplified using primers A36R-5' (5'-CCGGAATTCATATGATGCTGGTACC-3') and A36R-3' (5'-GCGGGATCCC ACCAATGATACG-3'); the A56R gene was amplified using primers A56R-5' (5'-CCGGAATTCATATGACACGATTACC-3') and A56R-3' (5'-GCGGGATCCGACTTTGTTCTCTG-3'); the B5R gene was amplified using primers B5R-5' (5'-GCATCAGCCCCATATGAAAACGATTTCCGTT GTTACGTT-3') and B5R-3' (5'-GCGGGATCCCGGTAGCAATTTATGG-3'); the F13L gene was amplified using primers F13L-5' (5'-GCATC AGCCCCATATGTGGCCATTTGC-3') and F13L-3' (5'-GCGGGATCCAATT TTTAACG-3'). All the PCR products were digested with restriction enzymes *Nde*I and *Bam*HI and ligated into *Nde*I/*Bam*HI-digested pTM1:L5R-6xHis, producing plasmids pTM1:A14L-6xHis, pTM1:A22R-6xHis, pTM1:A33R-6xHis, pTM1:A34R-6xHis, pTM1:A36R-6xHis, pTM1:A56R(COP)-6xHis, pTM1:A56R(IHD-J)-6xHis, pTM1:A56R(WR)-6xHis, pTM1:B5R-6xHis, and pTM1:F13L-6xHis.

Transient expression of the candidate palmitylproteins was facilitated by use of the vTF7-3 recombinant VV. BSC_{40} cells were grown to confluency on 35-mm wells in 6-well tissue culture plates (10^6 cell/well). The cells were infected with vTF7-3 at a moi of 10. Following a 1-h virus adsorption period, the inoculum was aspirated and replaced with 500 µl of MEM-E LG/GS containing 10 µg of plasmid DNA complexed with the transfection reagent DMRIE-C (Gibco-BRL). At 4 hpi (also 4 h post-transfection) an additional 500µl of MEM-E, LG/GS was added. If label was to be added it was added with the 500 µl of MEM-E, LG/GS at 4 hpi. At 24 hpi the cells were washed free of the tissue

culture plates using the culture medium. The cells were pelleted from the medium by centrifugation at 700 x *g* for 10 min. The cells were resuspended in 100 µl of PBS containing 5 U of DNase I to reduce the viscosity of the cell lysate, following lysis by 3 cycles of freezing and thawing, to facilitate gel loading. Eighty µl of each of the cell lysates were set aside for immunoprecipitations. The remaining 20 µl of cell lysates were made 1X reducing SDS-PAGE sample buffer by addition of 4 µl of 6X reducing SDS-PAGE sample buffer before loading gels.

2.2.7 Immunoprecipitation of Proteins

Eighty-µl samples of infected cell lysates (approximately $8.0x10^5$ cells), labeled during infection with [^{3}H]-PA, were diluted to 1 ml with radio-immunoprecipitation assay (RIPA) buffer (1% w/v sodium deoxycholate, 1% v/v Triton X-100, 0.2% w/v SDS, 150 mM sodium chloride, 50 mM Tris-HCl, pH 7.4). Histidine-tagged proteins were immunoprecipitated by addition of an anti-6-histidine monoclonal antibody (Qiagen) and Protein A-coated sepharose beads. The reactions were performed on a rotary mixer at 4°C for 18 h. The immunoprecipitates were washed numerous times with RIPA buffer then resolved by SDS-PAGE. The gels were fluorographed in PPO:DMSO and exposed to film for detection.

2.2.8 Mutagenesis of Palmitate Acceptor Residues in VV Palmitylproteins

Oligonucleotide (oligo)-directed mutagenesis of VV palmitylprotein genes on pTM1:6xHis plasmids followed the method of Kunkel *et al*(51). All plasmids resulting from the mutagenesis reactions were identical to their parental plasmids with the exception of the mutated codon and novel restriction sites engineered for preliminary screening. The restriction sites did not alter the coding potential of the genes. All mutations were confirmed by DNA sequencing. The codon encoding cysteine 99 (C99, numbering from the initiating methionine) of A22R was mutated to encode serine using oligo A22R-C99S (5'-GCCAAAG TGATCAGCGTCTCGCCTGTC-3') producing plasmid pTM1:A22R-C99S. The codon encoding C36 of A33R was mutated to encode serine using oligo A33R-C36S (5'-GTGATTGGTCTATCGATTAGAATATCT ATGG-3') producing plasmid pTM1:A33R-C36S. The codon encoding C19 of A36R was mutated to encode serine using oligo A36R-C19S (5'-GGAACAATACTAGTATCTTATATATTATATATTTGTAGG-3') producing plasmid pTM1:A36R-C19S. The codon encoding C25 of A36R was mutated to encode serine using oligo A36R-C25S (5'-GTTATATATTATATATCTCGAGGAAAAAGATACG-3') producing plasmid pTM1:A36R-C25S. The codon encoding C301 of B5R was mutated to encode serine using oligo B5R-C301S (5'-GTTATAGTA TTAGTCTCGAGTTGTGACAAAAATAATGAC-3') producing plasmid pTM1:B5R-C301S. The codon encoding C303 OF B5R was mutated to encode serine using oligo B5R-C303S (5'-GTTATAGTATTAGTTTGC TCGAGTGACAAAAATAATGAC-3') producing plasmid pTM1:B5R-C303S. An internal region of the F13L ORF on pTM1:F13L-6xHis containing codons for C185 and C186 was replaced with the homologous sequence from pDG5.3 (33) (see Chapter 3). This plasmid contains a copy of F13L but harbors mutations leading to the replacement of C185/C186 with serines without altering the otherwise wild-type sequence. This produced plasmid pTM1:F13L-C185/6S. No mutations were made to the A56R plasmid. Instead,

clones of A56R from COP, IHD-J and WR DNA were made. The COP and WR sequences of the A56R gene are known (32, 92). The IHD-J A56R DNA was sequenced for comparison to WR and COP. All plasmids were sequenced through the cloned genes to ensure accuracy of the sequence.

2.3 Results

2.3.1 Analysis of VV Acylproteins

VV potentially encodes more than 200 proteins expressed in three distinct temporal classes, early, intermediate and late. To limit the number of proteins to target for our study we performed a time-course labeling experiment with [^{3}H]-PA and [^{3}H]-MA to determine their temporal class. BSC_{40} cells were grown to confluency on 35-mm wells in 6-well plates. They were then synchronously infected with the COP and WR strains of VV at an moi of 10. The cells were co-infected so that all VV palmitylproteins and myristylproteins could be detected at the same time. COP does not produce the 92-kDa myristylprotein, but produces an 86-kDa protein not present in WR-infected cells. The infected cells were labeled by addition of [^{3}H]-PA or [^{3}H]-MA at 100 μCi/ml from 0-1, 1-2, 2-4, 4-8 and 8-24 hpi. The infected cells were harvested at the end of each labeling period. The total cell extracts were resolved by SDS-PAGE followed by fluorography and then exposed to film. With the exception of a 14-kDa protein that incorporated label from both myristic and palmitic acids in the 1-2 hpi labeling period, all other acylproteins encoded by VV appear to be "late" proteins (Figure 2.1). The 14-kDa acylprotein continued to incorporate label from 2-8 hpi but was nearly absent in the 8-24 hpi labeling period. All other VV acylproteins incorporated label in the 2-4, 4-8 and 8-24

Figure 2.1. SDS-PAGE analysis of time-course labeled VV acylproteins. BSC_{40} cells were co-infected with WR and COP strains of VV at a combined moi of 20. Cells were labeled with [^{3}H]-PA (A and B) or [^{3}H]-MA (C and D) for time periods as indicated above the gel lanes (hours post-infection). Identical gels were treated with neutral hydroxylamine to cleave thioester-bonded fatty acids (B and D). All gels were fluorographed by impregnation with PPO prior to film exposure. Note the 14-kDa acylprotein recalcitrant to hydroxylamine treatment in B and D.

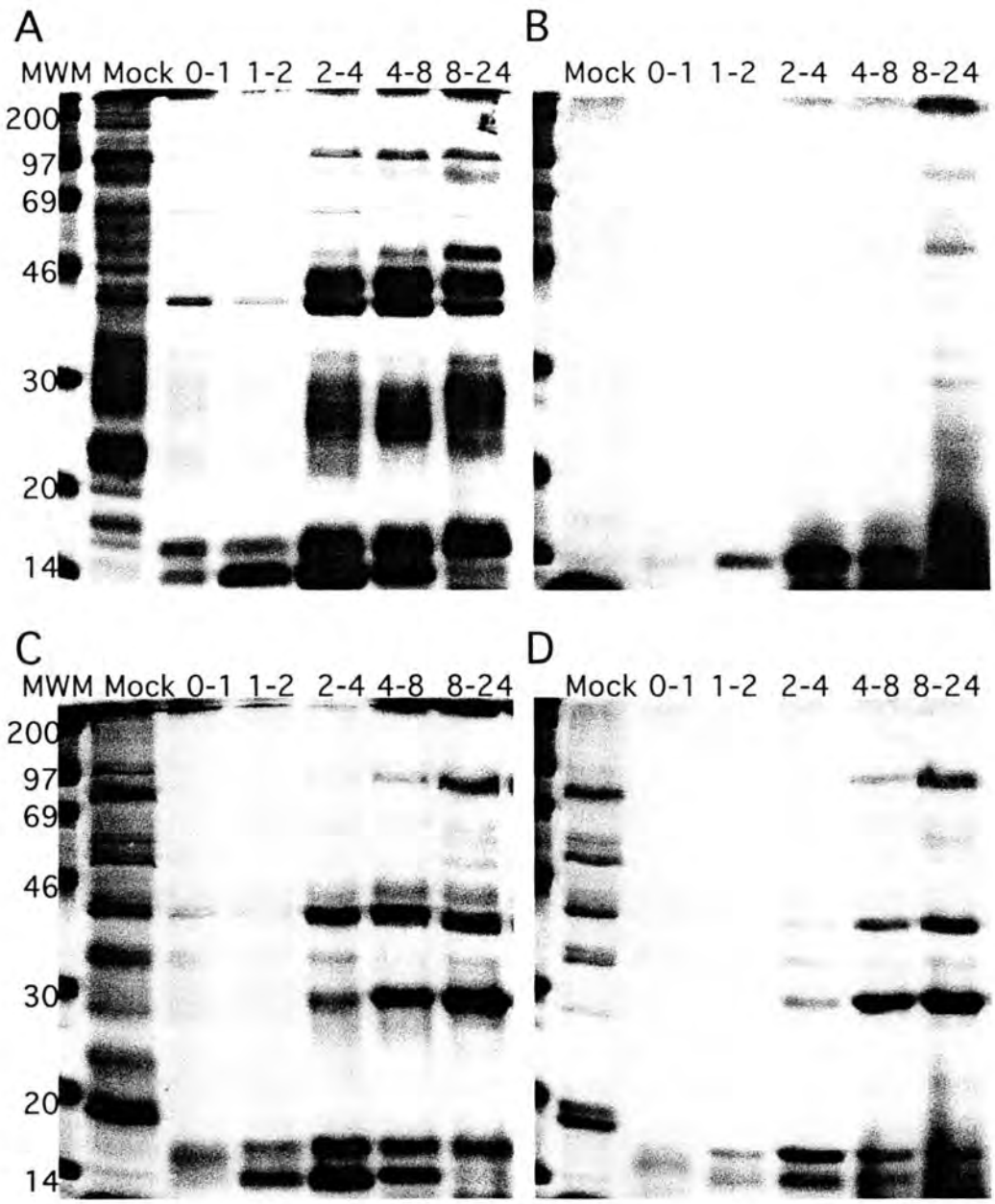
A
MWM Mock 0-1 1-2 2-4 4-8 8-24
200
97
69
46
30
20
14
B
Mock 0-1 1-2 2-4 4-8 8-24
C
MWM Mock 0-1 1-2 2-4 4-8 8-24
200
97
69
46
30
20
14
D
Mock 0-1 1-2 2-4 4-8 8-24

Figure 2.1

hpi labeling periods. To distinguish between authentic myristylproteins and those that were labeled through interconversion to palmitic or other long-chain fatty acids, identical gels were treated with hydroxylamine before fluorography (Figure 2.1C). Vaccinia encodes palmitylproteins with apparent molecular masses of 14, 17, 23-28 (a single glycoprotein with a common peptide backbone migrating as three distinct species), 37, 42, 46, and 94 kDa (Figure 2.1A). Additionally, the COP strain produced a 86-kDa protein that was not detectable in the WR or IHD-J infected cell extracts (Figure 2.2A). The 23-28-, 37-, and 42-kDa proteins have previously been reported to be encoded by the A33R (81), F13L (40) and B5R (45) ORFs respectively. The identities of the 14-, 17-, 46-, 86-, and 94-kDa palmitylproteins are unknown and represent the topic of this chapter.

The myristylproteins migrated with apparent molecular masses of 14, 17, 25, 36, 39, and 92 kDa (Figure 2.1D). The 92-kDa myristylprotein was not produced by the COP strain (Figure 2.2A) and most likely is the ATI protein encoded by the A25L ORF of WR and IHD-J as previously reported (31, 59). The 25-kDa myristylprotein is encoded by the L1R ORF and has been the subject of numerous studies (29, 71, 72, 111). We have recently reported that the 17-, 36-, and 39-kDa proteins are encoded by the E7R, A16L and G9R ORFs respectively (57). In a single report, the 14-kDa myristylprotein was identified as the product of the A14L ORF (77) - a finding that is not supported by our work (see section 2.3.6 and Figure 2.6).

2.3.2 Subcellular Fractionation and Virion Association of VV Acylproteins

The L1R myristylprotein is known to be a component of both IMV and EEV (70, 72). The A33R (81), B5R (45, 109) and F13L (9)

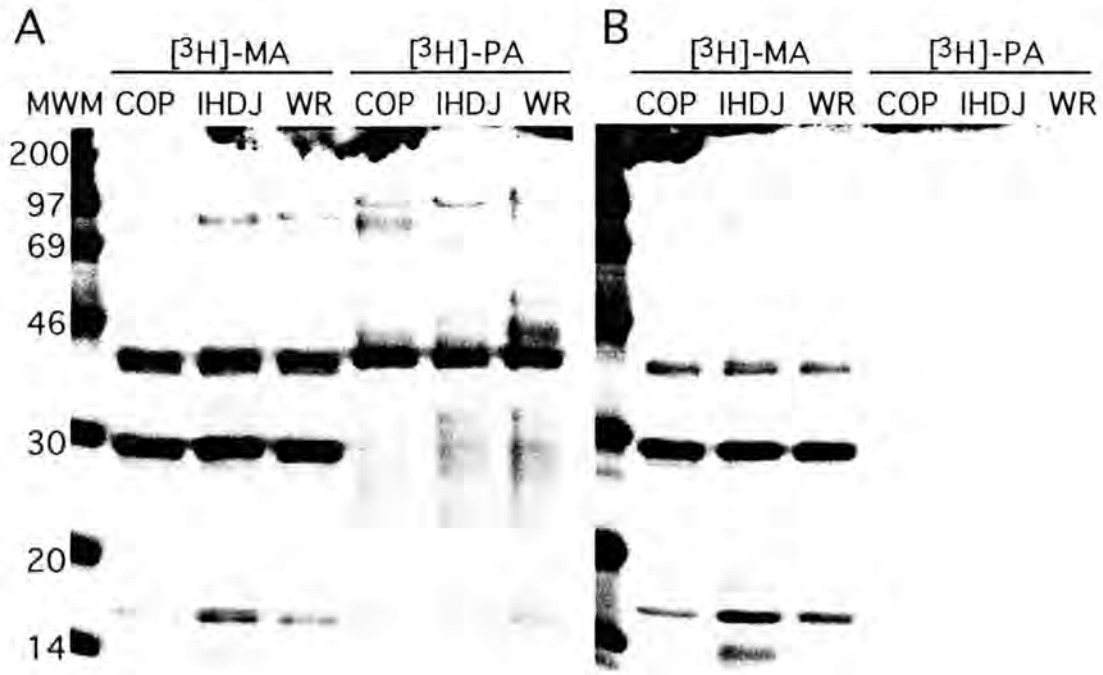

Figure 2.2. Comparison of acylproteins encoded by COP, IHD-J and WR strains of VV. BSC_{40} cells were infected with COP, IHD-J or WR strains of VV at an moi of 10. Cells were labeled by addition of [^{3}H]-PA or [^{3}H]-MA from 4 to 24 hpi. Total cellular extracts were resolved by SDS-PAGE and acylproteins were detected by exposure of PPO-impregnated gels to film (A and B). An identical gel was treated with neutral hydroxylamine to cleave thioester-bonded fatty acids (B). Note the absence of a 92-kDa myristylprotein ([^{3}H]-MA) from COP extracts. Also note the presence of an 86-kDa palmitylprotein in COP extracts that is absent from IHD-J and WR extracts.

palmitylproteins are known to be specific for TGN membrane-enveloped virions (IEV, CEV and EEV) and are found in TGN membranes prior to envelopment (84). To determine the intracellular location of the other acylproteins, we fractionated infected, labeled cells by differential centrifugation to yield a nuclear fraction, particulate cytosolic fraction (containing whole virions and cytosolic membranous components) and soluble

fraction. We also purified IMV and EEV from labeled cells. The L1R myristylprotein was present in the nuclear fraction and the membrane fraction, which also contains whole virions (Figure 2.3B). It was also associated with IMV and EEV. All other myristylproteins were found in the soluble fraction of cells although a small amount of the G9R protein is present in IMV also. All VV palmitylproteins found in total cell extracts were present in the nuclear fraction and the membrane fraction and were specific for EEV (Figure 2.3A). Two additional palmitylproteins with apparent molecular masses of 20 and 22 kDa, which were not obvious in the total cell extracts, were present on EEV as well. No VV palmitylproteins were found to be soluble or IMV-associated.

2.3.3 Identification of Candidate Palmitylproteins

To identify candidate palmitylproteins we considered that VV palmitylproteins are of the late temporal class, are specific for IEV, CEV and EEV, and most likely contain hydrophobic sequences mediating membrane association. We then scanned the sequence of the COP strain (which is entirely sequenced) (32) for ORFs preceded by late promoters and encoding proteins whose predicted mass corresponded to SDS-PAGE observed mobility of VV palmitylproteins. These sequences were then analyzed for hydrophobic regions and for cysteines that were within the consensus, Hydro*1-12*AACA*. By these criteria the following ORFs were identified as potentially encoding palmitylproteins: A14L, A22R, A33R, A34R, A36R, A56R, B5R and F13L (Figure 2.4). It has been previously reported that the products of the A33R (81), B5R (45) and F13L (38, 40) ORFs are palmitylproteins and led us to conclude that our criteria were sufficient. Additionally, the products of the A33R (81), A34R (23), A36R (62), A56R (68), B5R (25, 45), and F13L (38, 40) ORFs are specific for IEV, CEV and EEV further supporting these as candidate palmitylproteins.

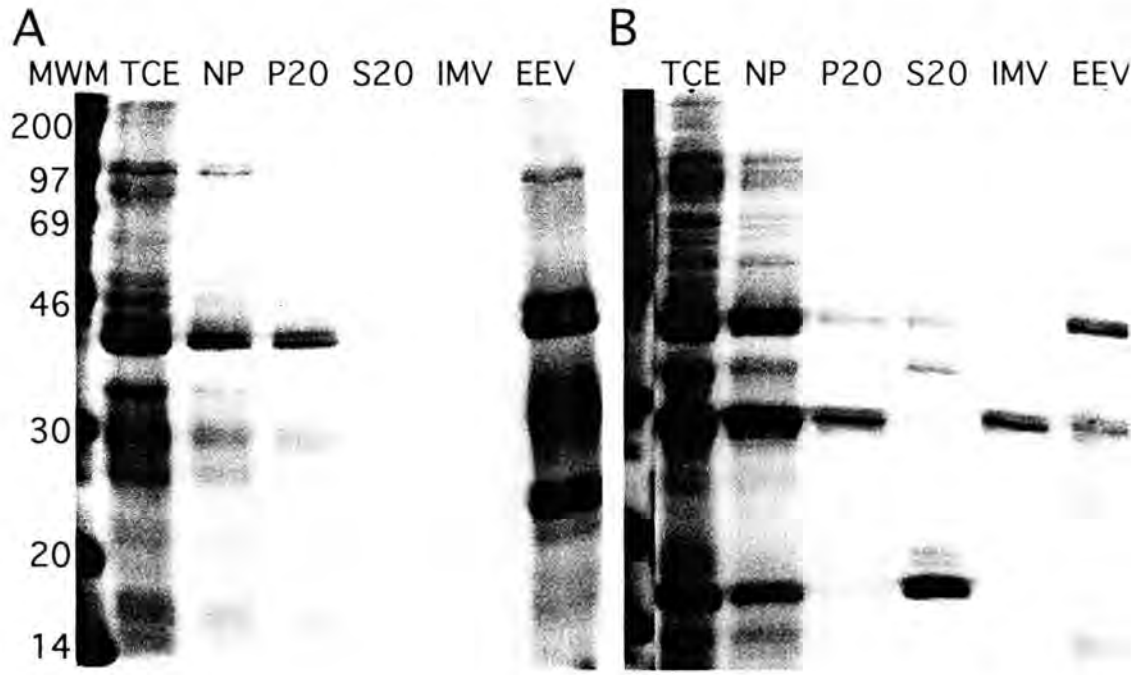

Figure 2.3. Differential centrifugation subcellular fractionation of VV acylproteins. BSC_{40} cells were co-infected with COP and WR strains of VV at a combined moi of 20. Cells were labeled with [^{3}H]-PA (A) or [^{3}H]-MA (B) from 4 to 24 hpi. EEV was purified from the culture medium. A portion of the total cell extracts (TCE) was fractionated to yield a nuclear fraction (NP), a particulate cytosolic fraction (P20), containing intracellular membranes and whole virions, and a soluble cytosolic fraction (S20). IMV was purified from infected cells. Fractions and equal titers of IMV and EEV were resolved by SDS-PAGE. Gels were impregnated with PPO prior to film exposure to detect [^{3}H]-PA-or [^{3}H]-MA-labeled proteins.

Gene	Promoter	Peptide Sequence	Size
A14L	ATTTAAAATTTTTATTAGTTAAATG	IAGIILLILSC*IFAFIDFSKS	10-kDa(15)
A22R	TAAATAACC(35)CAATCTTTAATG	LYHTSAAKVIC*VSPVMSGNSY	21-kDa(21)
A33R	GATATAAATAACATTTATTATCATG	KNKRKRVIGLC*IRISMVISLL	21-kDa(23-28)
A34R	ATTTATTTTTGTACATTAATAAATG	HYKEELMPSAC*ANGWIQYDKH	20-kDa(21-24)
A36R	TAAATA(9)TAAATA(31)GAAATG	VIVVAGTILVC*YILYIC*RKK	25-kDa(45-50)
A56R	ATAAATCACTTTTTATACTAATATG	LLILSAVAIFC*ITYYIC*NKR	35-kDa(72-86)
B5R	TTTTTAACACTCATAAATAAAAATG	IFLISVIVLVC*SC*DKNNDQY	35-kDa(42)
F13L	TATAAGTTTTTATGTTAACTAAATG	SWLNLC*SAAC*C*LPVSTAYH	41-kDa(37)

Figure 2.4. Identification of candidate VV palmitylproteins. Using a conceptual translation of the VV genome (COP strain), VV proteins were evaluated as candidates for palmitylation. Proteins whose promoters contain the consensus "late" start sequence upstream of the start ATG (bolded) were considered as candidates. Late promoter elements are underlined. Numbers in parentheses indicate the number of nucleotides that separate the nucleotides shown. The A56R gene does not contain a consensus "late" promoter but is included for additional reasons (see text). The peptide sequences of VV late proteins were inspected for homology to the palmitylation motif Hydro*1-12*AACA*. Proteins containing hydrophobic regions (underlined) with cysteine residues in an aliphatic environment (C*) were considered for further analysis. The predicted size of the protein is compared to the SDS-PAGE observed mobility (numbers in parentheses under "Size").

2.3.4 Transient Expression and Palmitylation of Candidate Palmitylproteins

The ORFs for each candidate palmitylprotein were PCR amplified from VV genomic DNA and cloned into a modified pTM1 vector, pTM1:6xHis. This vector contains sequences that, following expression, result in the addition of a carboxy-terminal 6-histidine tag on each of the candidate palmitylproteins. The T7 promoter and encephalomyocarditis virus 5' untranslated region (EMC leader) are left intact as well. Following infection of cells with vTF7-3 (a T7 polymerase-expressing recombinant vaccinia) the T7 promoter is recognized by the virus-produced T7 polymerase mediating high-level transcription. The EMC leader present on the transcript serves as an internal ribosomal entry site mediating cap-independent translation. For these experiments, 10^6 BSC_{40} cells were infected with vTF7-3 at an moi of 10 followed by transfection of 10 μg of each of the plasmid DNAs in separate wells of 6-well plates (35-mm wells). At 4 hpi [^{3}H]-PA was added to a final concentration of 100 μCi/ml in the culture medium. At 24 hpi the infected cells were harvested and resuspended in 100 μl of PBS. Ten μl of each sample was resolved by SDS-PAGE on two identical gels. The first gel was electroblotted to nitrocellulose and probed with Ni^+-activated horseradish peroxidase, which binds to the 6-histidine tag on the transiently expressed proteins. The blot was then developed by chemiluminescent reagents and exposure to film. The other gel was fluorographed in PPO:DMSO, dried and exposed to film for detection of all [^{3}H]-PA-labeled proteins present in the total cell extracts. The remaining 80 μl of sample was immunoprecipitated by using an anti-6-histidine monoclonal antibody. The immunoprecipitated proteins were resolved by SDS-PAGE. The gels were fluorographed in PPO:DMSO, dried and exposed to film as above.

The affinity blot (referring to the Ni+-activated horseradish peroxidase-probed blot) demonstrated that all the proteins were efficiently expressed by the vTF7-3/pTM1:6xHis system (Figure 2.5A). We observed the following: A14L encodes a protein of 14 kDA with minor species migrating at approximately 16 and 22 kDa; A22R encodes a 21-kDa protein; A33R encodes three proteins migrating at 23, 25, and 28 kDa; A34R encodes two proteins migrating at 22 and 24 kDa; A36R encodes a protein poorly resolving between 45 and 50 kDa; A56R encodes possibly three proteins resolving between 72 and 86 kDa; B5R encodes a 42-kDa protein and is present in a higher molecular weight complex of approximately 94 kDa; and F13L encodes a 37-kDa protein. The fluorograph of the same cell extracts, labeled with [^{3}H]-PA, demonstrate that the A14L, A34R and A56R (IHD-J and WR strains) proteins did not incorporate the label above background levels, suggesting that they are not palmitylproteins (Figure 2.5B and 2.5C). Proteins expressed from A22R, A33R, A36R, A56R (COP strain), B5R, and F13L incorporated the label significantly above background levels, suggesting that these ORFs encode palmitylproteins. It has been previously demonstrated that the A33R, B5R and F13L genes encode palmitylproteins, while A22R, A36R and A56R (COP strain) are newly discovered palmitylproteins.

2.3.5 Identification of Palmitate-Modified Residues of VV Palmitylproteins

To narrow the number of residues that might be subject to palmitylation, each of the VV palmitylprotein sequences was examined for cysteine residues that fell within the context of the palmitylation motif, Hydro*1-12*AACA*. We also considered that those regions of proteins exposed to the lumenal compartment of intracellular organelles or the extracellular environment were unlikely to be palmitylated due to the reducing nature of those

environments. By these analyses it was predicted that the following residues (denoted by the single-letter amino acid code and residue number starting from the initiating methionine) were palmitylated: C99 of A22R, C36 of A33R, C19 or C25 of A36R, C296 or C301 of A56R, C301 or C303 of B5R, and C185 and/or C186 of F13L. The codons for these residues were mutated to encode serine instead of cysteine by oligo-directed mutagenesis. In the case of F13L, both cysteine 185 and 186 were mutated to serine since it has been previously demonstrated to be palmitylated on both residues (34). Also, since we have observed that the COP-encoded A56R is palmitylated but is not palmitylated as expressed from the WR and IHD-J strains, their sequences were analyzed for differences occurring in the region predicted to be palmitylated. The only difference noted was that C301 from COP A56R was replaced by a tyrosine residue in both WR and IHD-J. Tyrosine is not known to be an efficient substrate for palmitylation, suggesting that C301 was the palmitate acceptor residue in that protein.

The mutated proteins were transiently expressed and analyzed by SDS-PAGE as above (Figure 2.5). The C99 mutant of A22R still incorporated label as efficiently as the wild-type protein, perhaps even more efficiently. The C36 mutant of A33R also incorporated label but at a slightly lower level than the wild-type protein. This may be due to differences in expression levels though and as such C36 may not be the palmitate acceptor residue. The C19 mutant of A36R incorporated label as efficiently as the wild-type protein, while the C25 mutant incorporated very little, suggesting that C25 is the primary palmitate-acceptor residue for A36R. The C301 mutant of B5R incorporated very little label, suggesting that C301 is the primary palmitate-acceptor residue. The C303 mutant of B5R incorporated approximately half that of the wild-type protein mutant, suggesting possibly a secondary modification site. The C185/186 mutant of F13L did not incorporate label at detectable levels above background in agreement with our previous report (34).

Figure 2.5. Transient expression and [^{3}H]-PA labeling of VV candidate palmitylproteins. Candidate palmitylproteins were transiently expressed in BSC_{40} cells by using the vTF7-3/pTM1:6xHis system. Proteins were labeled by addition of [^{3}H]-PA to infected cells from 4 to 24 hpi. Total cellular extracts were resolved on two identical gels (A and B). One gel (A) was blotted to nitrocellulose and probed with Ni^+-activated horseradish peroxidase, which binds histidine-tagged proteins. The blot was developed using chemiluminescent reagents and exposed to film. The other gel (B) was impregnated with PPO and exposed to film to detect all palmitylproteins in the cellular extracts. Histidine-tagged proteins were immunoprecipitated from the same cellular extracts using an anti-6-histidine monoclonal antibody. Immunoprecipitates were resolved by SDS-PAGE (C). The gel was impregnated with PPO and exposed to film to detect immunoprecipitated palmitylproteins. Gel lane assignments are identical for all three gels and are indicated above gel "A".

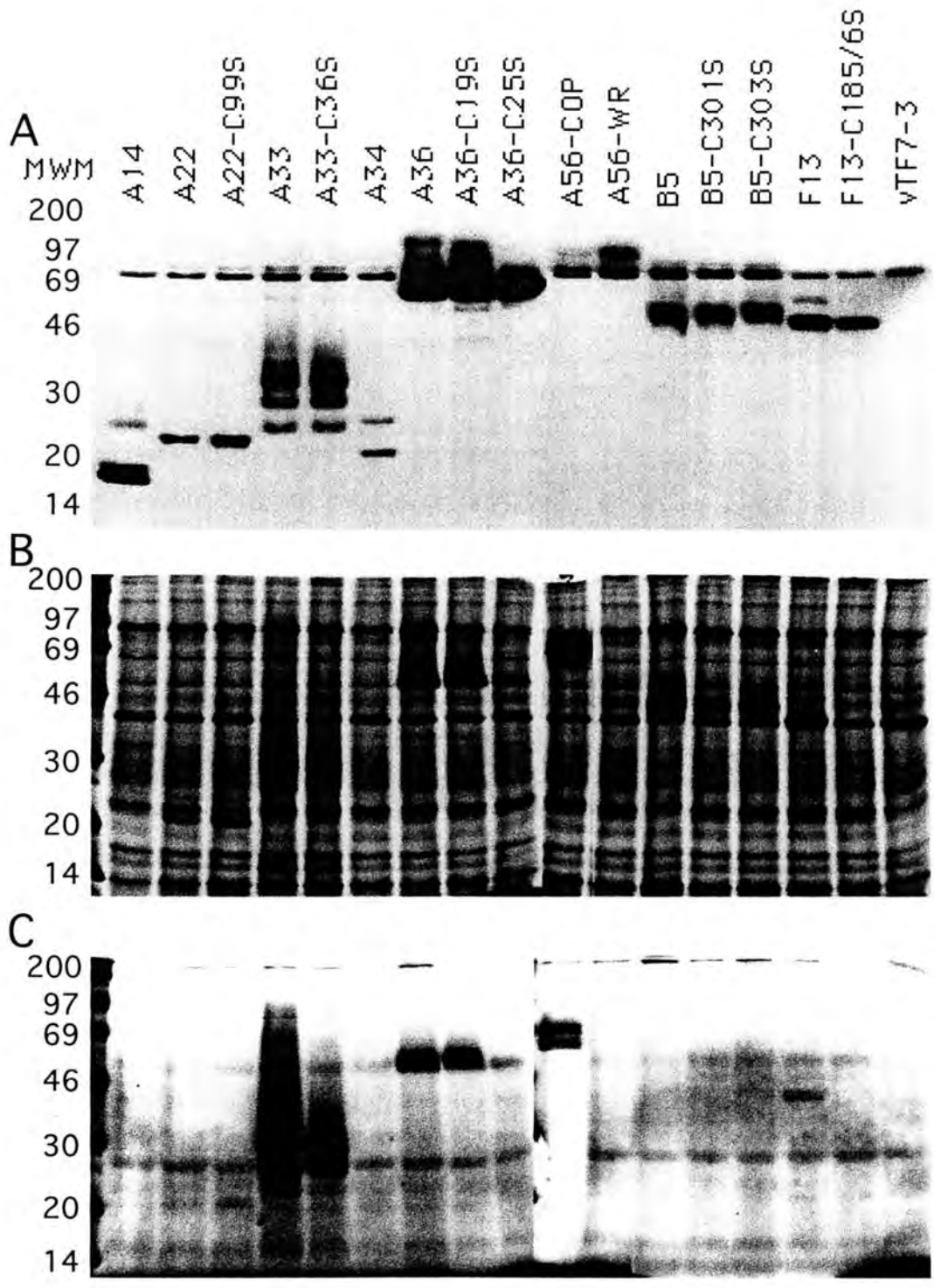
A
MWM
A14
A22
A22-C99S
A33
A33-C36S
A34
A36
A36-C19S
A36-C25S
A56-COP
A56-WR
B5
B5-C301S
B5-C303S
F13
F13-C185/6S
vTF7-3
200
97
69
46
30
20
14
B
200
97
69
46
30
20
14
C
200
97
69
46
30
20
14

Figure 2.5

2.3.6 [^{3}H]-MA Labeling of the A14L Protein

It has been previously reported that the A14L protein is myristylated (77). We attempted to confirm this by transient expression of the A14L protein in the presence of [^{3}H]-MA, using the vTF7-3/pTM1:6xHis system. Although the protein was efficiently expressed (Figure 2.6A), it did not incorporate the label (Figure 2.6B). We noticed, though, that overexpression of the A14L protein inhibited the production of other late VV myristylproteins while the intermediate 14-kDa myristylprotein was still expressed at a low level (Figure 2.6B).

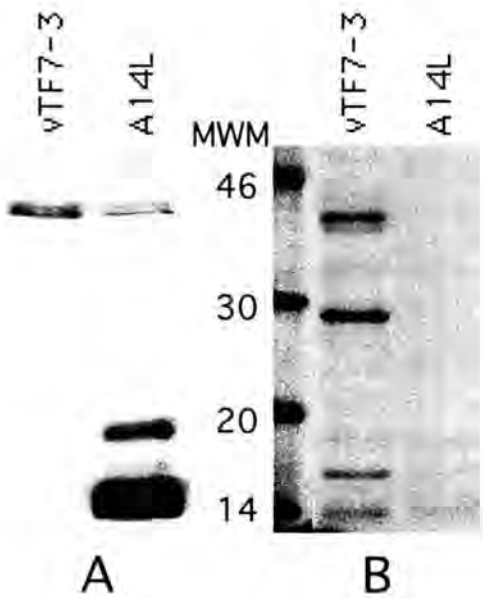

Figure 2.6. [^{3}H]-MA labeling of the VV A14L protein. BSC40 cells were infected with vTF7-3 and transfected with pTM1:A14L-6xHis. Cells were labeled by addition of [^{3}H]-MA from 4 to 24 hpi. Total cellular extracts were resolved by SDS-PAGE on two identical gels. One gel was blotted to nitrocellulose and probed with Ni$^+$-activated horseradish peroxidase (A). The gel was developed by using chemiluminescent reagents and exposed to film. The other gel was impregnated with PPO and exposed to film to detect myristylproteins (B).

2.4 Discussion

In identifying candidate proteins for this study, a number of criteria were considered. First, we considered the work of others to identify both viral and cellular palmitylproteins and their sites of modification (8, 20, 46, 50, 53, 69, 90, 105). With this information we were able to perform an alignment of numerous palmitylproteins using the palmitylated cysteine as a reference point. In doing so, we observed a loosely conserved motif that included a hydrophobic element that could be a previously acylated residue (such as a myristylated glycine), a transmembrane domain or a hydrophobic stretch of amino acids. This preceded the palmitylated cysteine by a 1- to 12-amino acid spacer region. Aliphatic amino acids usually surround the palmitylated cysteine. The essential elements in this motif are summarized by the acronym, Hydro*1-12*AACA* (34, 35).

This motif by itself did not allow the identification of VV palmitylproteins. Therefore, as a second method of identifying VV palmitylproteins, we undertook a biochemical characterization of palmitylated proteins expressed in VV-infected cells. We first made an effort to distinguish between VV myristylproteins and VV palmitylproteins, many of which co-migrate when resolved by SDS-PAGE. One of our first discoveries was that the ATI protein, which we previously thought to be modified by both myristate and palmitate (56), is in fact only myristylated. This was determined by the analysis of [^{3}H]-MA- or [^{3}H]-PA-labeled extracts from cells infected with the COP, IHD-J or WR strains of vaccinia. We observed an 86-kDa palmitylprotein that was expressed by COP was absent from the extracts from IHD-J- or WR-infected cells (Figure 2.2). A 94-kDa palmitylprotein was present in the extracts from all three viruses. In the [^{3}H]-MA-labeled extracts we observed a 92-kDa myristylprotein encoded by IHD-J and WR but not COP. It is known that the COP strain contains a mutation in the A25L gene encoding the ATI protein, that results in the premature

termination of translation, producing a truncated peptide (32, 63). Considering, though, that COP encodes palmitylproteins of 86 and 94 kDa it is likely that, due to their similar migration rates in SDS-PAGE gels, the 94-kDa palmitylprotein (also expressed by WR and IHD-J) and the 92-kDa myristylprotein were considered to be the same. Our conclusion that the ATI protein is not palmitylated is further supported by the finding that with the exception of L1R, all VV myristylproteins are soluble in the cytoplasm of infected cells while all VV palmitylproteins are found in the particulate fraction of infected cells. A remote possibility exists that the ATI protein is palmitylated and once it is it becomes a component of the particulate fraction of cells. This does not seem likely though because a 92-kDa palmitylprotein present in extracts from WR and IHD-J but not COP has not been observed.

Also, in comparing the acylproteins expressed by COP, IHD-J and WR strains of VV, we noted that COP encoded an 86-kDa palmitylprotein whose migration pattern in SDS-PAGE gels suggested that it was a glycoprotein. We also observed what appeared to be the same protein present on EEV. It is known that the VV hemagglutinin protein (encoded by gene A56R) exhibits similar characteristics so we considered it a candidate for palmitylation. Conceptual translation of the COP A56R gene suggested that either C296 or C301 would be possible palmitate acceptor residues. C296, although surrounded by aliphatic residues, is within the predicted transmembrane region. C301 is at the membrane/cytosolic boundary but is followed by the charged amino acid, asparagine. We sequenced the IHD-J A56R gene and compared it to the WR and COP sequences. The amino acid sequences from the transmembrane domain through the cytoplasmic tails for all three proteins were identical, with position 301 being the exception. While COP had a cysteine at position 301, both IHD-J and WR had a tyrosine residue. Considering that we have observed the COP hemagglutinin to be palmitylated while the IHD-J and WR hemagglutinin are not, we must conclude that C301 is the palmitate acceptor residue. This

conclusion is supported by the work of Payne (65) in which he demonstrated that the IHD-J hemagglutinin was not palmitylated.

Mammalian poxvirus hemagglutinin proteins are highly conserved. All strains of VV and variola have identical sequence from the transmembrane domain through the carboxy terminus of the protein with the exception of two positions. All strains of VV have a threonine at position 288, while all strains of variola have an alanine. The other variable position is amino acid 301. All strains of variola, plus the COP and Wyeth strains of VV, have a cysteine at position 301, while VV strains IHD-J, WR, LIVP, Tian Tan, and MVA have a tyrosine at the same position. The significance of the variability at this position is not clear to us.

As part of our biochemical characterization of VV acylproteins, we performed a time-course labeling experiment to determine the temporal class to which the VV acylproteins belong. All VV myristyl- and palmitylproteins are expressed primarily at late times, suggesting a role in assembly. The one exception is the 14-kDa acylprotein that incorporated label from both myristic and palmitic acids. We are led to conclude that they are the same protein due to identical expression characteristics and size. Expression of both the myristic and palmitic acid-labeled 14-kDa acylprotein began in the 1- to 2-hpi labeling period, continued through the 2- to 8-hpi labeling period, and was not detectable in the 8- to 24-hpi labeling period. The possibility that both the myristylated and palmitylated proteins are the same is supported by the fact that a fraction of the label, whether added as myristic or palmitic acid, incorporated by the 14-kDa protein was recalcitrant to treatment by neutral hydroxylamine (Figure 2.1B and 2.1D) which is known to cleave thioester-linked moieties such as palmitate (83). This suggests that at least part of the label incorporation was due to the formation of neutral hydroxylamine-stable amide linkage as is typical of myristylation. Vaccinia is not predicted to encode a 14-kDa protein containing the amino-terminal myristylation motif MG*XXX*S/T/A/C/N, and this

therefore suggests that the 14-kDa protein is the second example of VV-encoded internally myristylated protein, the first being the ATI protein. It has been previously reported that the A14L gene encodes a 14-kDa myristylprotein (77). We attempted to confirm this and to determine whether this protein was palmitylated as well. Overexpression of the A14L gene in the presence of [^{3}H]-PA or [^{3}H]-MA did not result in the incorporation of either label, although the protein was expressed at a high level (Figure 2.5 and 2.6). We did note, though, that the high-level expression of A14L inhibited other late protein production. The conclusion that the A14L protein is myristylated was based on a single experiment in which the A14L protein was immunoprecipitated from myristic acid-labeled infected cell extracts. A 14-kDa protein was precipitated. It may be that the A14L protein interacts with the 14-kDa myristylprotein and was thus co-precipitated, leading to the conclusion that the A14L protein was myristylated. We have observed a similar phenomenon when precipitating A14L from palmitic acid labeled infected cell extracts. A 14-kDa palmitylprotein is co-precipitated but migrates with a slightly different migration pattern from A14L, allowing us to distinguish between the two.

We have not only identified novel palmitylproteins encoded by the A22R, A36R and A56R (COP strain) genes of VV, but we have been able to accurately predict the palmitate acceptor residue for A36R-, A56R-, B5R- and F13L-encoded proteins. Although, by our analyses, VV proteins appear to be palmitylated in a predictable manner, the misidentification of the A14L and A34R proteins as candidate palmitylproteins and the inability to predict the modification sites on the A22R and A33R proteins suggest shortcomings to our system for identification of VV palmitylproteins. Perhaps additional criteria should be considered. The A14L protein is known to be a component of the IMV membrane and the inner membrane of EEV. Although the primary amino acid sequence indicates that it could be a substrate for palmitylation, its presence in the IMV membrane may sequester it from the

palmitylation machinery. It is not clear to us why a protein in the membrane of IMV would not be palmitylated while a protein in the membrane of EEV would be. Another factor that must be considered is membrane orientation. The A36R protein is predicted to be a type II membrane protein and as such, the most likely palmitate acceptor residue would be exposed to the lumenal environment of intracellular membrane-bound compartments or to the extracellular environment if it was exported to the plasma membrane. These environments are reducing and exposure would likely result in the cleavage of the thioester-bonded palmitate. Our finding that the A36R protein is palmitylated on C25 suggests the opposite membrane orientation, with a short amino-terminal lumenal tail and a long carboxy-terminal cytoplasmic tail. This is supported by a recent finding by Röttger *et al* (82) that the carboxy-terminal region of the protein is cytoplasmic. Membrane orientation may explain why the A34R protein is not palmitylated. The A34R protein is predicted to be a type II transmembrane protein with a long, lumenally exposed carboxy-terminal tail. The predicted orientation appears to be correct based on the findings of Röttger *et al.* Even though the primary amino acid sequence is an exact match for what we define as an optimal consensus for palmitylation, the presence of the motif in the lumenal compartment prevents palmitylation.

In the report by Röttger *et al*, the type II membrane orientation for the A33R protein was confirmed. Only one cysteine residue (C36) is present in the cytoplasmic domain of the protein. When C36 is changed to serine by oligo-directed mutagenesis, the A33R protein still incorporated [^{3}H]-PA label, although at slightly reduced efficiency. Serine was chosen to replace cysteine due to similar biochemical characteristics such as size and the polar side chain. In retrospect, this may not have been the best choice. Serine and threonine are also substrates for palmitylation but only rarely (8, 89). It may be that replacement of a palmitate-acceptor cysteine with a serine in an optimal environment results in palmitylation of the serine residue through ester linkage.

Many VV palmitylproteins are thought to interact either through disulfide bonds or noncovalently (65, 82). The exact nature of these interactions or their biological significance is not known. These proteins are also involved in the formation of IEV, the precursor of EEV, the propulsion of the IEV particle through and out of cells by actin polymers, or in the infectivity of released virions. They are all specific for the TGN-enveloped virions and prior to virion envelopment are found associated with the membranes of the TGN (84). It is not likely a coincidence that many of these proteins are palmitylated. It may be that the palmitate moiety targets these proteins to their respective intracellular location, i.e., the TGN. Alternatively, it may be that the palmitate moiety stabilizes hydrophobic protein/protein interactions. We have previously reported that, at least for the F13L protein (p37), the palmitate modification is necessary for TGN association (33, 34) (see Chapter 3). A mutant of p37 that is not palmitylated is soluble in the cytoplasm of infected cells and cannot function efficiently regarding envelopment of IMV. It will be of interest to determine the biological significance of the palmitylation of the other VV palmitylproteins.

VV continues to stand out as an excellent system for the study of mammalian protein processing. VV proteins (or exogenous proteins expressed either transiently from transfected plasmids or from recombinant VV) are subject to modifications typical of mammalian systems, to include sulfation, phosphorylation, ADP-ribosylation, glycosylation and, as highlighted here, acylation. The ease with which VV is genetically manipulated has facilitated the study of numerous VV gene products, leading to the identification of three new VV palmitylproteins.

Chapter 3

Analysis of a Vaccinia Virus Mutant Expressing a Nonpalmitylated Form of p37, a Mediator of Virion Envelopment

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3.1 Introduction

Vaccinia virus (VV) is a member of the *Poxviridae*, a family of large, complex DNA viruses that replicate in the cytoplasm of infected cells (61). Its nearly 200 kilobasepair genome has been completely sequenced and appears to encode more than 200 gene products (32). While expressing many of the enzymes and cofactors required for replication and expression of its genome, including numerous nucleotide metabolizing enzymes, the virus is dependent on the host cell translational machinery for protein production. Proteins are expressed in discrete temporal classes, starting with the early class as soon as 15 minutes after virus entry into the cell. The intermediate class of genes is expressed from about 2 to 4 h post-entry, followed by the late class, which is expressed from 4 h post-entry until cell death. Virion assembly coincides with late protein production.

The first immature virions appear (by electron microscopy) as hollow membrane crescents that loosely surround the core components. The first infectious virions, referred to as IMV, are formed as the core, composed of the genome, packaged enzymes and co-factors, and numerous structural proteins, condenses. The envelope surrounding the IMV particle, most likely derived from the intermediate compartment between the endoplasmic reticulum and the Golgi apparatus (93), but possibly produced *de novo* by virus-specific membrane forming machinery, also contains virus-encoded proteins (41). In tissue culture systems, IMV represent the majority of virions produced, but the *in vivo* significance of this virion form remains to be established. Depending on the strain of virus and host cell, 25-40% of the total IMV produced are enveloped with additional membranes and released from the cell (66). These multiply enveloped forms of the virus are likely of greater significance *in vivo* as they have been implicated in the cell-to-cell spread and long-range dissemination of the virus (67).

The envelopment of IMV is a complex process that involves targeting of the IMV particle to the TGN, where, by budding through the compartment, two additional surrounding membranes are acquired, forming IEV (38). The IEV particle uses actin polymerization to propel itself through and out of the cell, sometimes into neighboring cells without exposure to the extracellular environment (10, 17, 37, 39, 96). At the plasma membrane, the outermost envelope of the IEV particle fuses with the cell membrane releasing virions to the outside of the cell with the loss of the outermost membrane. If the virion remains attached (or reattaches) to the cell it is referred to as CEV and when free-floating in the extracellular medium it is referred to as EEV.

The formation of infectious enveloped virions is dependent on numerous VV-encoded proteins including the products of the A27L (76), A33R (80), A34R (12, 58, 110), A36R (62), B5R (26, 109), and F13L (encoding p37) (9) ORFs of vaccinia. All of these except for the product of A27L, a 14-kDa IMV-associated protein, are specific for the enveloped forms of the virus and are located in the outermost envelope of EEV. The A34R gene product is glycosylated (23), while the A33R and B5R gene products are both glycosylated and palmitylated. The p37 protein is palmitylated but is not glycosylated (65). The A36R protein has only recently been demonstrated to be a palmitylprotein (see Chapter 2) and its migration pattern in SDS-PAGE gels suggests glycosylation.

Fatty acylation has been demonstrated to be an important modification for VV-encoded proteins, contributing in more than one stage of virion assembly. In addition to numerous myristylproteins (27), vaccinia also encodes at least eight palmitylated proteins of 14, 17, 21, 23-28 (a single protein), 37, 42, 45-50 (a single protein), 86 (COP strain only), and 94 kDa (16, also see Chapter 2). All but the identities of the 14- and 17-kDa proteins are known. The 21-kDa protein is encoded by the A22R ORF. The 23-28-kDa protein (gp23-28) is the glycosylated product

of the A33R gene previously thought to be encoded by the A34R gene (84). The 37-kDa protein is p37 (40), the product of the F13L gene and the focus of this chapter. The 42-kDa protein (gp42) is the glycosylated product of the B5R gene (45). The 45-50-kDa protein (gp45-50) is encoded by the A36R open reading frame. The 86-kDa protein (gp86, the VV hemagglutinin) is encoded by the A56R ORF of the COP strain of VV. The 94-kDa protein is a multimeric complex composed of the B5R protein and possibly others. The site(s) of modification is (are) known for gp45-50, gp86, gp42 and p37.

p37 is expressed from 4 hpi until cell death. Its electrophoretic mobility indicates a relative mass of 37 kDa, but its predicted mass, based on amino acid content, is 41 kDa. Within infected cells, it is targeted to the TGN (38), and when virion associated, is specific for the enveloped forms of the virus (36, 64). Hydrophobicity and membrane topology predictions suggest that it is a transmembrane protein, based on a hydrophobic alpha-helical region in the central part of the protein. Recent biochemical studies argue against the predictions and indicate the protein is a peripheral membrane protein (91). The significance of the fatty acyl moiety in anchoring the protein to membranes has been demonstrated by two methods. The protein normally fractionates with the membranous components of infected cells and cannot be stripped from membranes by salt or carbonate extraction. If the membranes are treated with neutral hydroxylamine, which cleaves the thioester bonded palmitate moiety from the protein, the protein is released from the membranes (91). Indirect immunofluorescent analysis of cells in which p37 is transiently expressed demonstrates that the wild-type protein is present in discrete cytoplasmic foci, while a nonpalmitylated mutant is evenly diffused throughout the cytoplasm (34).

Recent evidence suggests that p37 may be a member of the phospholipase D superfamily, all of which contain a motif

(H*X*K*XXXX*D using the single-letter amino acid code) also present in p37 (98). Mutation of this motif in p37, or other phospholipase D superfamily members, results in loss of function, either in the envelopment and release of virus (p37) or enzymatic (human and yeast phospholipase D). Baek *et al* have demonstrated that p37 exhibits phospholipase A and C activity rather than phospholipase D activity. The *in vivo* substrates for p37 are unknown. It has been suggested that the products of the lipase reactions may be involved in membrane fusion.

While these studies have contributed to our understanding of p37 function and protein-membrane biochemistry, they have not answered the fundamental question, "Why is p37 palmitylated?". In this chapter we have attempted to answer that question by the construction and analysis of a mutant virus that expresses a nonpalmitylated p37 rather than the wild-type fatty acylated protein. Our findings indicate that the biological significance of the acyl moiety is in targeting p37 to intracellular membranes whereby the protein is functional in cooperatively enveloping IMV.

3.2 Materials and Methods

3.2.1 Cells and Viruses

BSC_{40} and RK13 cell lines were cultured in MEM-10 LG/GS at 37°C in a 5% CO_2 humidified atmosphere. Upon infection with VV, the cells were then cultured in Eagle's MEM LG/GS at 37°C in a 5% CO_2 humidified atmosphere. The IHD-J strain of VV was routinely propagated and titered in BSC_{40} cells as previously described (24). It has not been genetically altered and encodes the wild-type p37. The mutant virus vRB10 has been previously described (9). In vRB10, 93% of the F13L ORF encoding p37 has

been deleted by insertion of the mycophenolic acid resistance gene, *gpt,* under transcriptional control by the VV 7.5 kDa promoter. It was propagated by low-multiplicity passage through BSC_{40} cells in the presence of mycophenolic acid, xanthine and hypoxanthine. Plaque titrations were performed by inoculating serial dilutions of the virus onto BSC_{40} monolayers followed by transfection of a plasmid-borne rescuing copy of the F13L gene behind a VV promoter. Plaques were visualized by crystal violet staining at 72 hpi.

3.2.2 Construction of Recombinant VV

The plasmid pVV5.1:*neo* (28) was restricted with *Eco*RI and *Xba*I to release a 1192-basepair fragment containing the entire bacterial neomycin phosphotransferase II (NEO) gene (Figure 3.1). The NEO gene was ligated into *Eco*RI-/*Nhe*I-restricted pRB21 (11), placing the gene downstream from a VV synthetic early/late promoter. The resulting plasmid is referred to as pDG5.0. The plasmid pDG4.3, previously demonstrated to contain an engineered mutation in the F13L ORF resulting in the expression of a nonpalmitylated form of p37 (34), was restricted with *Spe*I and *Kpn*I to release a 672 base pair internal fragment of the F13L ORF. This fragment was ligated into *Spe*I-/*Kpn*I-restricted pDG5.0 replacing the wild-type F13L ORF with the mutated copy. This plasmid is referred to as pDG5.3. In this plasmid, the codons for the normally palmitylated cysteines (cysteines 185 and 186) are mutated to code for serine instead. The presence of the mutated copy of the F13L ORF was confirmed by restriction digestion with *Hind III* as there is an additional site in the mutated gene not found in the wild-type sequence and by DNA sequencing.

The plasmids were transfected into BSC_{40} cells simultaneously infected by vRB10 as follows: Ten μg of plasmid DNA (either pDG5.0 or pDG5.3) was added to 1 ml of MEM LG/GS in

Figure 3.1. Diagram of the construction of recombinant viruses, vWTp37 and vPA$^-$p37. Plasmids used or constructed in this study: pRB21, pVV5.1:*neo*, pDG5.0, pDG4.3, pDG5.3. Vaccinia promoters are indicated by a boxed "P". Protein-coding regions are indicated for each plasmid. F13L encodes p37, *neo* encodes neomycin phosphotransferase II, gpt encodes hypoxanthine-xanthine guanine phosphoribosyltransferase. The nucleotide sequences flanking the F13L open reading frame in the vaccinia genome are indicated by F13L+L and F13L+R. The mutated F13L gene sequence and the nonpalmitylated p37 protein it encodes are indicated by an asterisk (*).

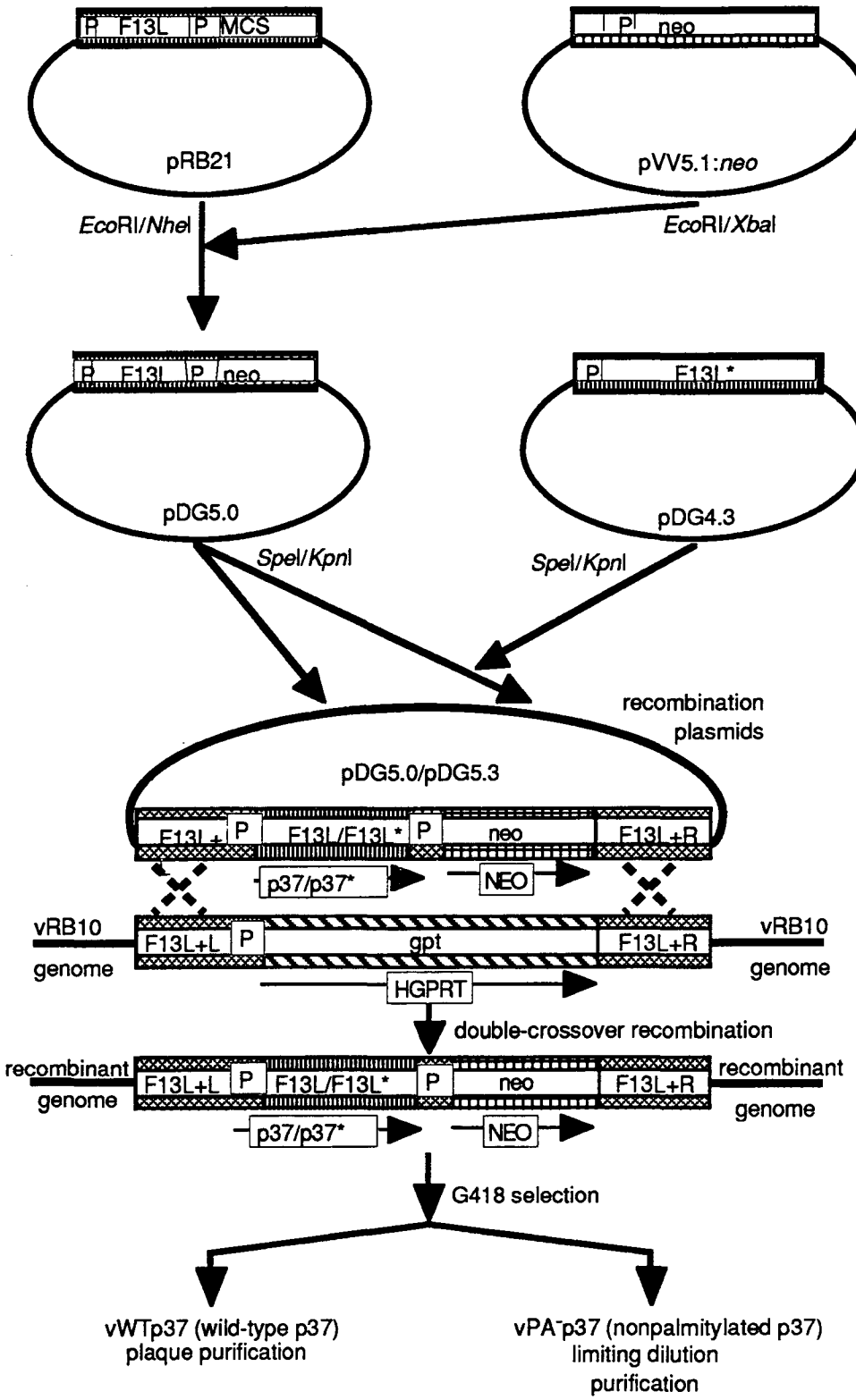
P F13L P MCS
pRB21
P neo
pVV5.1:neo
EcoRI/NheI
EcoRI/XbaI
P F13L P neo
pDG5.0
P F13L*
pDG4.3
SpeI/KpnI
SpeI/KpnI
recombination plasmids
pDG5.0/pDG5.3
F13L+L
P
F13L/F13L*
P
neo
F13L+R
p37/p37*
NEO
vRB10 genome
F13L+L
P
gpt
F13L+R
vRB10 genome
HGPRT
double-crossover recombination
recombinant genome
F13L+L
P
F13L/F13L*
P
neo
F13L+R
recombinant genome
p37/p37*
NEO
G418 selection
vWTp37 (wild-type p37) plaque purification
vPA-p37 (nonpalmitylated p37) limiting dilution purification

Figure 3.1

polystyrene tubes. Thirty µl of the liposome transfection reagent DMRIE-C (Life Technologies, Inc.) was added to each tube followed by incubation at room temperature for 15 min. After the DNA:liposome complexes had formed, $1x10^6$ infectious units of vRB10 were added to the mixture. The virus/DNA/liposome mixture was inoculated onto $1x10^6$ BSC_{40} cells in 35-mm wells of a 6-well tissue culture plate and incubated for 48 h at 37°C in a 5% CO_2 humidified atmosphere. At 48 hpi the infected cells were washed free of the tissue culture plate and suspended in the culture medium. Cells were pelleted from the suspension by centrifugation at 700 x *g*, 4°C, for 10 min. The cells were resuspended in 100 µl PBS and frozen, then thawed, 3 times to lyse cells and release virus. Ten µl of the infected cell lysates was used as an inoculum to infect $1x10^7$ BSC_{40} cells on 100-mm plates that had been pretreated with G418 at a concentration of 200 µg/ml in the culture medium for 24 h before infection. Upon infection, the G418 concentration was increased to 400 µg/ml in the culture medium (MEM LG/GS). The infection continued for 48 h at 37°C in a 5% CO_2 humidified atmosphere. The cells were then scraped free from the plate and resuspended in the culture medium. The cells were pelleted from the culture medium by centrifugation at 700 x *g*, 4°C, for 10 min. The cells were resuspended in 1 ml of PBS and frozen, then thawed, 3 times to lyse cells and release virus. One hundred µl of the infected cell lysates was used as an inoculum to infect BSC_{40} cells. The process of passing virus through G418-pretreated BSC_{40} cells in the presence of G418 was repeated 3 times to enrich for recombinant viruses expressing NEO.

After the third passage to enrich for recombinants, individual virions were amplified to obtain pure virus stocks. This was accomplished by two separate techniques. The recombinant virus expressing the wild-type p37, code named vWTp37, was purified by screening virus stocks grown from individual plaques formed in BSC_{40} monolayers overlaid with agar-containing medium plus 200 µg/ml G418. The expression of p37 by this virus

was confirmed as described below. The recombinant virus expressing the mutated and presumably nonpalmitylated form of p37, code named vPA$^-$p37, did not form normal-sized plaques (see Results below) so it could not be plaque purified as described for vWTp37. Instead, individual viruses were cultured by inoculating serial dilutions of the G418-passaged stocks onto BSC_{40} cells in 96-well tissue culture plates and treating with 200 μg/ml G418 in the culture medium as generally outlined by Blasco and Moss (9). At 72 hpi each well was examined microscopically to determine if microplaques were forming. Wells that had no plaques or more than one microplaque were disregarded. The cells from individual wells with only one detectable microplaque were scraped free from the plate with a pipette tip, resuspended in the culture supernatant, transferred to microcentrifuge tubes, and frozen, then thawed, 3 times. The entire cell lysate was used as an inoculum to infect $1x10^6$ BSC_{40} cells in 6-well tissue culture plates. The cells were pretreated with 200 μg/ml G418 for 24 h before infection and the infection was carried out in the presence of 200 μg/ml G418. At 24 hpi the cells were harvested and lysates prepared by freeze-thaw as described above. The lysates were screened for p37 production as described below and only those lysates that contained p37 were purified further. The p37-containing cell lysates were serially diluted and inoculated onto BSC_{40} cells in 96-well plates that had been pretreated with G418. Individual microplaques were observed microscopically at 72 hpi, harvested and amplified in the presence of 200 μg/ml of G418 as above. The limiting dilution purification of vPA$^-$p37 was performed a total of 3 times. Purified stocks of vWTp37 and vPA$^-$p37 were prepared as previously described (24) and stored at -70°C.

3.2.3 Sodium Dodecylsulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) and Immunoblot Detection of Proteins

Proteins from cell lysates or from purified virion samples were boiled in reducing sample buffer and resolved by discontinuous gel electrophoresis as described by Laemmli (52). All gels used were 12.5% polyacrylamide:bis-acrylamide (30:0.8). The proteins were transferred from gels to nitrocellulose (99) and sequentially probed with antibodies directed against VV antigens followed by enzyme-conjugated secondary antibodies. The blots were developed by addition of chemiluminescent substrates and exposure to film. All primary antibodies were from polyclonal antiserum produced in rabbits and have been described previously. Polyclonal antiserum directed against p37 (α–p37) (91) was used at 1:10,000 while antibodies directed against the 25-kDa VV core protein (α–25K) (108) and the IMV membrane protein encoded by the L1R ORF (α–L1R) (29) were used at 1:1000. The secondary antibody was a goat-anti-rabbit IgG polyclonal conjugated to horseradish peroxidase (GαR-HRP) diluted 1:40,000 for use. The antibody:protein complexes were detected by incubating the blots with SuperSignal® chemiluminescent substrate solutions (Pierce) followed by exposure to BioMax™ MR-2 film (Kodak).

3.2.4 [^{3}H]-Palmitic Acid Labeling of Virion Proteins and Immunoprecipitation of p37

BSC_{40} cells were grown to confluence (10^6 cells) in 35-mm wells of 6-well tissue culture plates. The cells were infected with IHD-J, vRB10, vWTp37 or vPA$^-$p37 at a moi of 10. At 4 hpi the media were aspirated and replaced with MEM LG/GS containing 200 μCi/ml [^{3}H]-PA. The infections continued until 24 hpi at 37°C in a 5% CO_2 humidified atmosphere. The cells were suspended in

the culture medium by repeatedly pipetting it over the cell monolayer. The cell suspensions were subjected to centrifugation at 12,000 x *g*, 4°C for 30 min to pellet cells and virus particles. The pellets were resuspended in 100 µl of PBS and frozen, then thawed, 3 times. Twenty µl was boiled in reducing SDS-PAGE sample buffer, and then equally divided between two 12.5% polyacrylamide gels. Following electrophoresis, one gel was impregnated with PPO:DMSO, dried and exposed to BioMax™ MR-2 film at -70°C (13). The other gel was transferred to nitrocellulose and p37 was detected by antibodies and chemiluminescence as above. The remaining 80 µl of infected cell lysates was diluted to 580 µl in 2X strength RIPA buffer and incubated on ice for 15 min. The solutions were then diluted to 1X concentration with water. One µl of α–p37 was added to each sample followed by incubation on ice for 2 h. Then, 40 µl of a 50% slurry of protein A-sepharose beads in 1X RIPA was added and incubation was continued for 18 h at 4°C with constant agitation. The immunoprecipitated proteins were washed three times with 1X RIPA, transferred to a new microcentrifuge tube and washed again. The beads were pelleted a final time and resuspended in reducing sample buffer and heated to 70°C for 1 min. The proteins were resolved by SDS-PAGE using 12.5% polyacrylamide gels as above. Following electrophoresis the gels were fluorographed by impregnation with PPO:DMSO, drying and exposure to BioMax™ MR-2 film at -70°C.

3.2.5 Plaque Formation and Infectious Titer Assays

Serial dilutions of purified virion preparations or infected cell lysates were made in MEM LG/GS and inoculated onto confluent monolayers of BSC_{40} cells in 35-mm wells of 6-well tissue culture plates. Under duplicate infection conditions one set of cells inoculated with vRB10 or vPA$^-$p37 dilutions was simultaneously transfected with pRB21 (a plasmid encoding wild-

type p37) to enhance plaque formation and facilitate determination of infectious titer for these samples. At 48 hpi, the cell monolayers were stained with 0.1% (w/v) crystal violet in 30% (v/v) ethanol and plaques observed both macro- and microscopically.

3.2.6 Metabolic Labeling and Purification of Virions

RK13 cells ($1x10^7$) were seeded onto 150-mm tissue culture plates and cultured in MEM-10 LG/GS at 37°C in a 5% CO_2 humidified atmosphere for 24 h. The cells were infected with either IHD-J, vRB10, vWTp37 or vPA$^-$p37 at moi of 10 and cultured in MEM LG/GS until 4 hpi at 37°C in a 5% CO_2 humidified atmosphere. The culture media were aspirated and replaced with methionine-deficient minimal essential medium containing 22.0 μCi/ml [^{35}S]-methionine/cysteine and LG/GS. The infection continued until 24 hpi. The culture supernatants were transferred to 15-ml tubes and centrifuged at 700 x *g*, 4°C for 15 min to pellet free-floating cells. The supernatants were transferred to centrifuge tubes and subjected to ultracentrifugation at 100,000 x *g*, 4°C for 1 h. The pellets were resuspended in 1 ml of PBS and plaque-assayed to determine infectious titers as described above.

Purification of virus from the infected RK13 cell lysates by sucrose gradients was as previously described (42). The cell monolayers were washed with PBS and then resuspended in 10 mM Tris-Cl pH 8.0. The cells were allowed to swell on ice for 15 min then Dounce homogenized to lyse them and release virus. The cellular debris was pelleted by centrifugation at 700 x *g*, 4°C for 15 min. The virus-containing supernatants were layered onto 6 ml 36% sucrose cushions and subjected to ultracentrifugation at 55,000 x *g*, 4°C for 80 min. The pellets were resuspended in 1 ml of 10 mM Tris-Cl pH 8.0 and Duall homogenized to disrupt aggregated material. They were then layered onto 25-40% sucrose

gradients and subjected to ultracentrifugation at 33,000 x *g*, 4°C for 40 min. The virus bands were side-pulled by using a needle and syringe to withdraw the entire band in no more than 1 ml of solution. Each virus sample was diluted to 1 ml with water and plaque-assayed to determine infectious titers as described above.

The remaining samples of extracellular and cell-associated virus were further purified by CsCl gradients (68). The gradients were prepared in ultracentrifuge tubes by first adding 5 ml of a 1.2 g/ml CsCl solution, followed by under-laying through Pasteur pipettes 4 ml of a 1.25 g/ml Cs Cl solution, then 2.5 ml of a 1.3 g/ml CsCl solution. The virus samples were subjected to ultracentrifugation at 100,000 x *g*, 15°C for 2 h. Five hundred-µl fractions were collected from the bottom of the gradients and the radioactivity per fraction was measured by scintillation counting. Peak fractions for IMV and EEV (as determined by relative position in the gradient) were pooled, diluted in water to 12 ml and subjected to ultracentrifugation at 100,000 x *g*, 4°C for 1 h. The virus pellets were resuspended in 100 µl of PBS and quantitated again by scintillation counting.

3.2.7 Proteinase K Treatment of Purified IMV and EEV

EEV and IMV samples, containing equivalent radioactivity, were treated with proteinase K at a final concentration of 2 µg/ml in PBS for 1 h at room temperature (91). Duplicate reactions were mock-treated. All reactions were stopped by addition of reducing SDS-PAGE sample buffer and boiling for three min. Samples were resolved by SDS-PAGE (12.5%) and blotted to nitrocellulose. The blots were probed with α–p37, and α–25K then stripped and probed with α–L1R. Chemiluminescent detection of proteins was as described above.

3.2.8 Electron Microscopy (EM)

BSC_{40} cells ($1x10^7$) in 100-mm tissue culture plates were infected with either IHD-J, vRB10, vWTp37 or vPA$^-$p37 at moi of 10. At 16 hpi the cells were scraped free from the plates and resuspended in the culture medium. The cells were pelleted by centrifugation at 700 x *g*, 4°C for 10 min. The cells were washed in PBS and pelleted again by centrifugation. The cells were fixed by resuspension in 2.5% paraformaldehyde (w/v): 200 mM cacodylate buffer, pH 7.4, and incubating at 4°C for 8 h. The cells were pelleted by centrifugation and washed once with 200 mM cacodylate buffer, pH 7.4. The cells were left in 200 mM cacodylate buffer pH 7.4 at 4°C for 16 h. The cells were pelleted by centrifugation. The cacodylate buffer was aspirated and replaced with a 1% osmium tetroxide(w/v) solution. The cell pellet was then incubated at room temperature for 1 h. The samples were dehydrated by sequential 15-min incubations in 25%, 50%, 75% and 100% acetone. The 100% acetone incubation was repeated three times. The samples were then infiltrated with Spurr's plastic. The samples were incubated at room temperature in solutions of 1/3 Spurr's:2/3 acetone for 4 h followed by 2/3 Spurr's:1/3 acetone for 16 h and finally 100% Spurr's for 6 h. The samples were placed under vacuum and heated to 70°C for 16 h. Sectioning was performed by personnel of the Oregon State University EM laboratory on a Sorvall Porter-Blum model MT2 ultramicrotome. Sections were post-stained by using Reynold's lead citrate and uranyl acetate before examination by transmission EM.

3.2.9 Indirect Immunofluorescent and Fluorescent Microscopy

BSC_{40} cells were seeded onto microscope slide cover slips ("microcover glasses", VWR) at a density of $5x10^5$ cells per 35-mm well in 6-well tissue culture plates and cultured 24 h at 37°C in a 5% CO_2 humidified atmosphere. The cells were infected with either IHD-J, vRB10, vWTp37 or vPA$^-$p37 at moi of 10. At 8 hpi the cells were rinsed with ice-cold PBS followed by 100% methanol. The cells were prepared for immunofluorescent microscopy as described by Watkins (107). The cells were fixed by incubating in 3.7% formalin for 20 min at room temperature followed by permeabilization with 0.2% Triton X100. The cells were rinsed twice for 5 min with ice-cold PBS. The cells were incubated with the primary antibodies α–p37 (used at a 1:2000 dilution) or α–L1R (used at a 1:500 dilution) in PBS containing 2% bovine serum albumin (BSA) as a blocking agent, for 1 h at 4°C. The cells were rinsed four times for 5 min each with ice-cold PBS. The secondary antibody was a goat-anti-rabbit IgG tetramethylrhodamine isothiocyanate conjugate (GαR-TRITC; Sigma) used at a 1:64 dilution in PBS containing 2% BSA. The secondary antibody incubations were for 1 h at 4°C in the dark. Forty min into the secondary antibody incubation, fluorescein isothiocyanate-conjugated phalloidin (Sigma) was added to a final concentration of 1 μg/ml. Twenty min later, the secondary antibody solution was aspirated and the cells washed four times for 5 min with ice-cold PBS. The final wash was aspirated and the cells allowed to air-dry for 10 min before mounting on slides and examination by confocal fluorescent microscopy.

3.2.10 Subcellular Fractionation

BSC_{40} cells were grown to be 95% confluent in 150-mm tissue culture plates ($2x10^7$ cells). The cells were infected with either IHD-J, vRB10, vWTp37, or vPA$^-$p37 at moi of 10. The infected cells were cultured in MEM LG/GS at 37°C in a 5% CO_2 humidified atmosphere for 16 h. The culture media were transferred to ultracentrifuge tubes and clarified by a 100,000 x *g*, 4°C, 1 h centrifugation. The cells were washed free of the plates with 10 ml of ice cold PBS. The cells were pelleted by centrifugation at 700 x *g*, 4°C for 10 min. The cells were fractionated using differential centrifugation essentially as described by Child and Hruby (16). The PBS was aspirated and the cells resuspended in 1.2 ml of hypotonic buffer (HB, 20 mM HEPES pH 7.6, 5 mM potassium chloride, 1 mM magnesium chloride, 150 mM sodium chloride) and incubated 10 min on ice to swell the cells. All subsequent steps were at 4°C. The cells were then lysed by Dounce homogenization. Two hundred µl of each cell lysate was set aside as the total cell extracts (TCE) while the remainder was subjected to centrifugation at 700 x *g* for 10 min. The supernatants (post-nuclear supernatant, PNS) were transferred to new microcentrifuge tubes for further fractionation while the pellets from that centrifugation were resuspended in 1.0 ml of HB and set aside as the nuclear pellet (NP). The PNS were transferred to centrifuge tubes and diluted to 4.5 ml with HB and subjected to ultracentrifugation at 100,000 x *g* for 60 min. The pellets from this centrifugation (P100) were resuspended in 1 ml of HB and set aside as the particulate cytoplasmic fractions. The supernatants from this centrifugation and the clarified culture supernatants were adjusted to 10% trichloracetic acid and subjected to centrifugation at 15,000 x *g* for 30 min. The pellets from this centrifugation were set aside as the soluble cytoplasmic fractions (S100) and the soluble culture supernatant fractions (SUP). All other fractions were adjusted to be 10% trichloracetic acid and subjected to centrifugation at 15,000 x *g* for 30 min.

With the exception of the TCE fractions, which were resuspended in 20 µl, all precipitated pellets were resuspended in 100 µl of 1 M Tris, pH 10 to neutralize the acid and briefly sonicated to facilitate resuspension. Twenty µl of each fraction was analyzed by SDS-PAGE (12.5% polyacrylamide gel) and immunoblot using α-p37 as the primary antibody. For chemiluminescent detection, the primary antibody incubation was followed by incubation with GαR-HRP, then SuperSignal® chemiluminescent substrate solutions followed by exposure to BioMax™ MR-2 film. Relative quantitation of protein:antibody complexes was performed by film densitometry.

3.3 Results

We have previously demonstrated that p37 is palmitylated on cysteine residues 185 and 186 of the 372 amino acid protein (34, also see Chapter 2). In addition we, along with others (91), have confirmed that it is the fatty acyl modification, and not the predicted transmembrane domain consisting of a hydrophobic alpha-helical region in the middle of the protein, that mediates membrane interaction by p37. These studies have been performed either *in vitro* or by transient expression of p37. To understand the biological significance of the fatty acyl modification in an *in vivo* context, we sought to construct a recombinant VV expressing a nonpalmitylated p37 rather than the wild-type fatty acylated protein. Once the recombinant was constructed, we then performed a series of assays designed to compare its activity to that of the wild-type virus, IHD-J. p37 is known to be involved in the process of envelopment and release of virions (9), so we tested for plaque formation (a process dependent on enveloped virions), release of virions from infected cells and retention of enveloped virions within infected cells. Also, the nucleation of actin to propel virions through and out of the cell is abrogated in the absence of p37 (17). It is not clear whether p37 is directly

involved in actin nucleation or merely a player in a prerequisite reaction. For that reason, we examined cells by fluorescent microscopy after fluorescently labeling VV antigens and F-actin. Also, to confirm our previous studies, we analyzed infected cells and virions for p37 localization to determine whether the palmitate moiety was important in targeting p37 to virions or intracellular membranes.

3.3.1 Construction of Recombinant Viruses

To facilitate the construction of the recombinants used in this study, two plasmids were made (Figure 3.1). One encodes the wild-type p37 (pDG5.0) and the other contains a mutated F13L sequence demonstrated to encode a nonpalmitylated p37 (pDG5.3) by transient expression (34). The plasmid pDG5.3 contains mutations that result in the replacement of the normally palmitylated cysteines (cysteines 185 and 186) with serines. The F13L ORF on both plasmids is transcriptionally regulated by the native F13L promoter and is flanked by sequences neighboring the F13L locus in the VV genome to allow for homologous recombination into its native site. The *neo* gene mediating G418 resistance, behind a synthetic VV early/late promoter, is also present on the plasmids to allow for selection of recombinants.

Cells were infected with vRB10, a virus that does not produce p37 due to an F13L deletion, and transfected with either pDG5.0 or pDG5.3. The progeny were passed numerous times in the presence of G418 followed by a plaque-purification of the wild-type p37-encoding virus, termed vWTp37, or a limiting dilution purification of the nonpalmitylated p37-encoding virus, termed vPA$^-$p37. The viruses were tested for expression and palmitylation of p37 by labeling infected cells with [^{3}H]-PA followed by immunoprecipitation of p37 from the cell extracts. The immunoprecipitates were resolved by SDS-PAGE and

Figure 3.2. Expression and palmitylation of p37. The tritiated palmitic acid-labeled extracts from cells infected with virus as indicated above the gel lanes in panel A were analyzed for p37 expression by immunoblot (A) using anti-p37 antiserum and chemiluminescent development of the blot. p37 was analyzed for incorporation of the labeled palmitic acid by immunoprecipitation (B), resolution by SDS-PAGE, and fluorography. The efficiency of labeled palmitate incorporation by other vaccinia proteins was demonstrated by SDS-PAGE and fluorography of the total infected cell extracts (C). Molecular weights are indicated beside each gel or blot. The previously described vaccinia palmitylproteins are indicated to the right of panel C. Unknown palmitylproteins are indicated by a "?". The 46-kDa and 28-kDa forms of p37 described in the text are indicated by enclosing p37 in parentheses (p37).

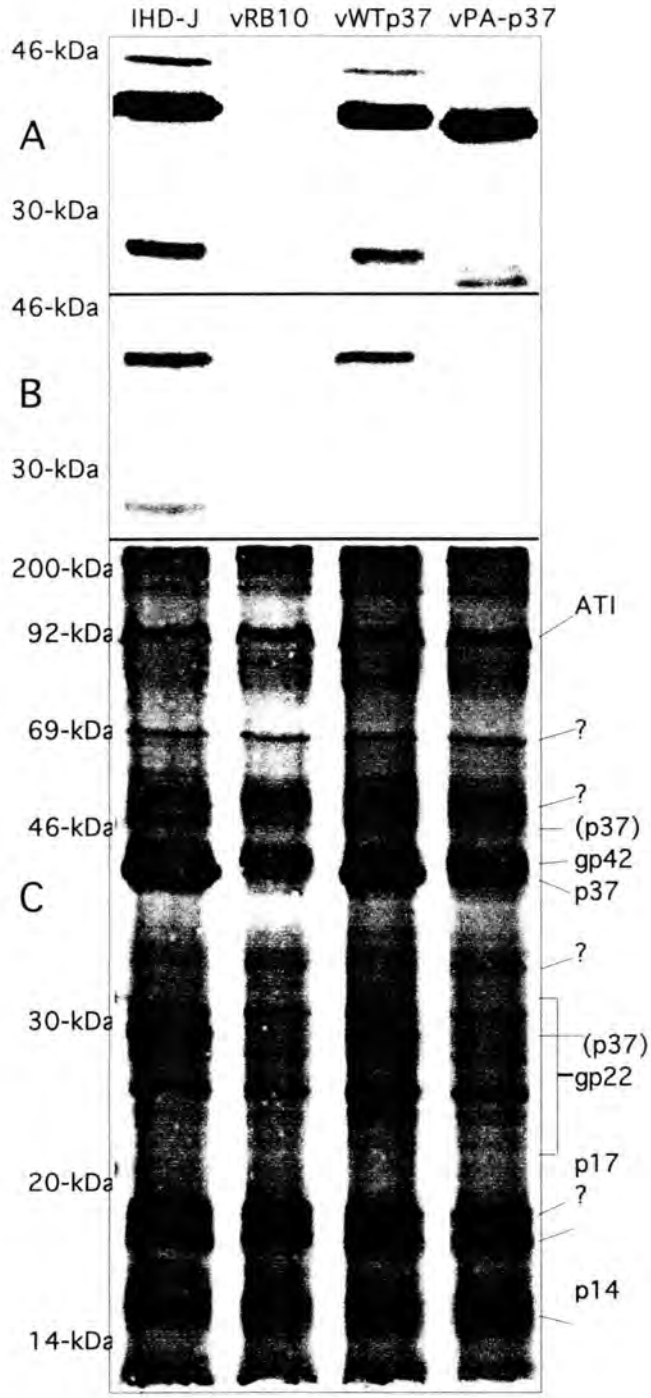
IHD-J
vRB10
vWTp37
vPA-p37
46-kDa
A
30-kDa
46-kDa
B
30-kDa
200-kDa
92-kDa
69-kDa
46-kDa
C
30-kDa
20-kDa
14-kDa
ATI
?
?
(p37)
gp42
p37
?
(p37)
gp22
p17
?
p14

Figure 3.2

fluorographed to detect label. The tritium label was incorporated by p37 expressed from IHD-J or vWTp37 (Figure 3.2B) but was not incorporated at detectable levels by vPA$^-$p37-expressed p37 although the immunoblots of the same cell extracts demonstrate that it was expressed at comparable levels (Figure 3.2A). We also noted that when total [^{3}H]-PA-labeled infected cell extracts were resolved by SDS-PAGE and fluorographed, there were three labeled protein bands in the extracts from IHD-J and vWTp37 that were absent from vRB10 and vPA$^-$p37 (Figure 3.2C). The same three bands were recognized by α–p37 in immunoblots suggesting that they all represent some form of p37. The 37 kDa band was the predominant form of the protein while the 46-kDa and 28-kDa forms are present in reduced amounts. We have concluded that both vWTp37 and vPA$^-$p37 express p37 at levels comparable to IHD-J and that vPA$^-$p37 encodes a nonpalmitylated p37. Furthermore, p37 is present in at least two additional forms other than the well-characterized 37-kDa form, although the faster migrating species may represent a degradation product, as we discuss below.

3.3.2 Formation of Enveloped Virions by vPA$^-$p37

The formation of IEV, and subsequently CEV and EEV, is dependent on p37 (9). The mutant virus vRB10 has had the F13L ORF insertionally inactivated and 93% deleted by a *gpt* expression cassette mediating mycophenolic acid resistance. As a result, it is inefficient at producing either enveloped virions or plaques on cell monolayers, for cell-to-cell spread is dependent on enveloped virions. The virus still produces normal amounts of infectious IMV, which are retained intracellularly unless released by cell lysis. Therefore, the ability to form plaques on cell monolayers stands as a simple test for enveloped virus production and release.

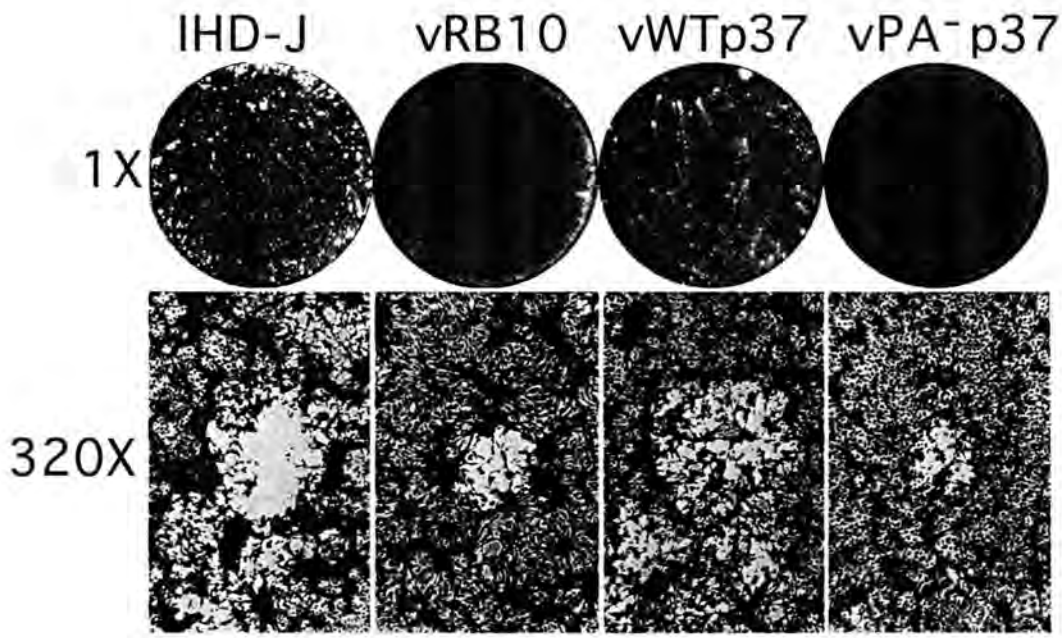

Figure 3.3. Plaque formation by wild-type and recombinant viruses. Monolayers of cells were infected at a low multiplicity of infection to allow observation of individual plaques. At 48 hours post-infection, the liquid overlay was aspirated and the cells stained with crystal violet. The infecting virus is indicated above the macroscopic view. Magnification is indicated to the left of both the macro- and microscopic views.

BSC40 cell monolayers were infected with serial dilutions of either IHD-J, vRB10, vWTp37 or vPA⁻p37 and cultured for 48 h. The cell monolayers were then stained with crystal violet to enhance visualization of the plaques. Both IHD-J and vWTp37 formed characteristic plaques with tails making them comet-like in appearance (Figure 3.3). This plaque morphology is the result of a single amino acid change in the A34R protein (12) and is typical of IHD-J and its derivative strains or mutants. Both vRB10 and vPA⁻p37 did not form visible plaques within 48 h although very small plaques were visible to the naked eye between 72 and 96 hpi. As confirmation that we had infected with vRB10 and vPA⁻p37, the cell monolayers were inspected microscopically. We were

able to see microplaques forming at 48 hpi but they were only approximately 1/10 the size of IHD-J or vWTp37 plaques (Figure 3.3).

To assure ourselves that inefficient enveloped virus production was a bona fide vPA$^-$p37 phenotype we used another system to assay enveloped virus production. It has been demonstrated that the amino acid substitution on the A34R protein leading to the production of comet-like plaques is also responsible for the enhanced release of EEV from RK13 cells (66). RK13 cells were infected with either IHD-J, vRB10, vWTp37 or vPA$^-$p37 at moi of 10 and cultured until 24 hpi. The virus purified from the culture supernatant, which at 24 hpi is almost 100% EEV, and the virus purified from cell extracts were separately tested for infectious titer by plaque assay. To enhance plaque formation by vRB10 and vPA$^-$p37, cells were simultaneously transfected with pRB21, a plasmid that transiently expresses p37 in VV-infected cells. All viruses produced roughly equivalent amounts of cell-associated virus (predominantly IMV). While there was a nearly 1:1 ratio of cell-associated virus to extracellular virus for IHD-J, vRB10 produced approximately 60- to 70-fold less extracellular virus (Figure 3.4). The 1:1 ratio is restored for vWTp37 as expected since the wild-type F13L gene has been reinserted into the vRB10 genome. vPA$^-$p37 remained defective for release of virus, producing from 33 to more than 100 times more cell-associated virus than extracellular virus.

Both envelopment assays employed above are dependent on the production and release of *infectious* virus. Mutants of VV have been described that release normal amounts of EEV but with very low infectivity, resulting in a small-plaque phenotype (58). We considered this a possibility for vPA$^-$p37, making necessary the quantitation of virion particles produced. RK13 cells were infected as above and labeled with [^{35}S]-methionine/cysteine after 4 hpi. At 24 hpi virions were concentrated from the culture medium and purified from cell lysates as described in Materials

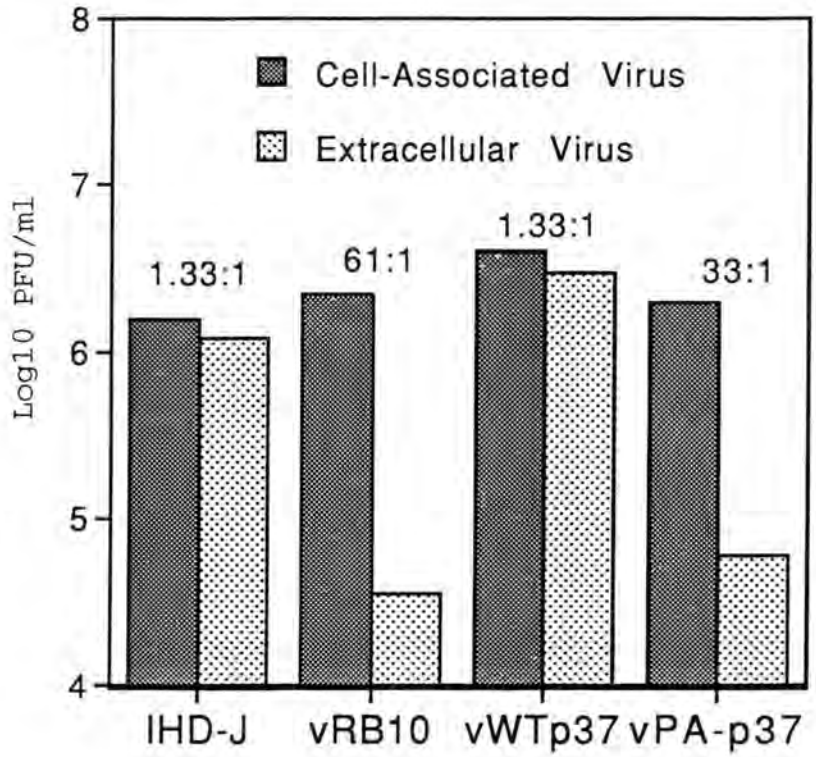

Figure 3.4. Comparison of virus production and release. RK13 cells were infected with virus as indicated on the "X" axis. At 24 hours post-infection virus was purified from cell lysates (cell-associated virus) and the culture supernatants (extracellular virus) and plaque titrated to determine infectious virus production. The ratio of cell-associated to extracellular virus is given above the bars for each virus.

and Methods. The labeled virus particles were purified by centrifugation in CsCl gradients that were then fractionated, followed by determination of radioactivity per gradient fraction. IMV is separable from enveloped virions due to differing buoyant densities (68). While all viruses produced roughly equivalent amounts of IMV as determined by radioactive peaks in the gradient at 1.26 to 1.27 g/ml (Figure 3.5B), IHD-J and vWTp37 had significantly higher peaks of radioactivity in fractions corresponding to densities at which enveloped virions sediment

Figure 3.5. Quantitation of IMV and EEV by metabolic labeling and CsCl gradient purification. Virus from cells (B) or the culture media (A) of RK13 cells, infected and labeled as outlined in Materials and Methods, was separated according to density by CsCl gradient centrifugation. Fractions were collected from the bottom of the gradients and radioactivity per fraction was quantitated by scintillation counting.

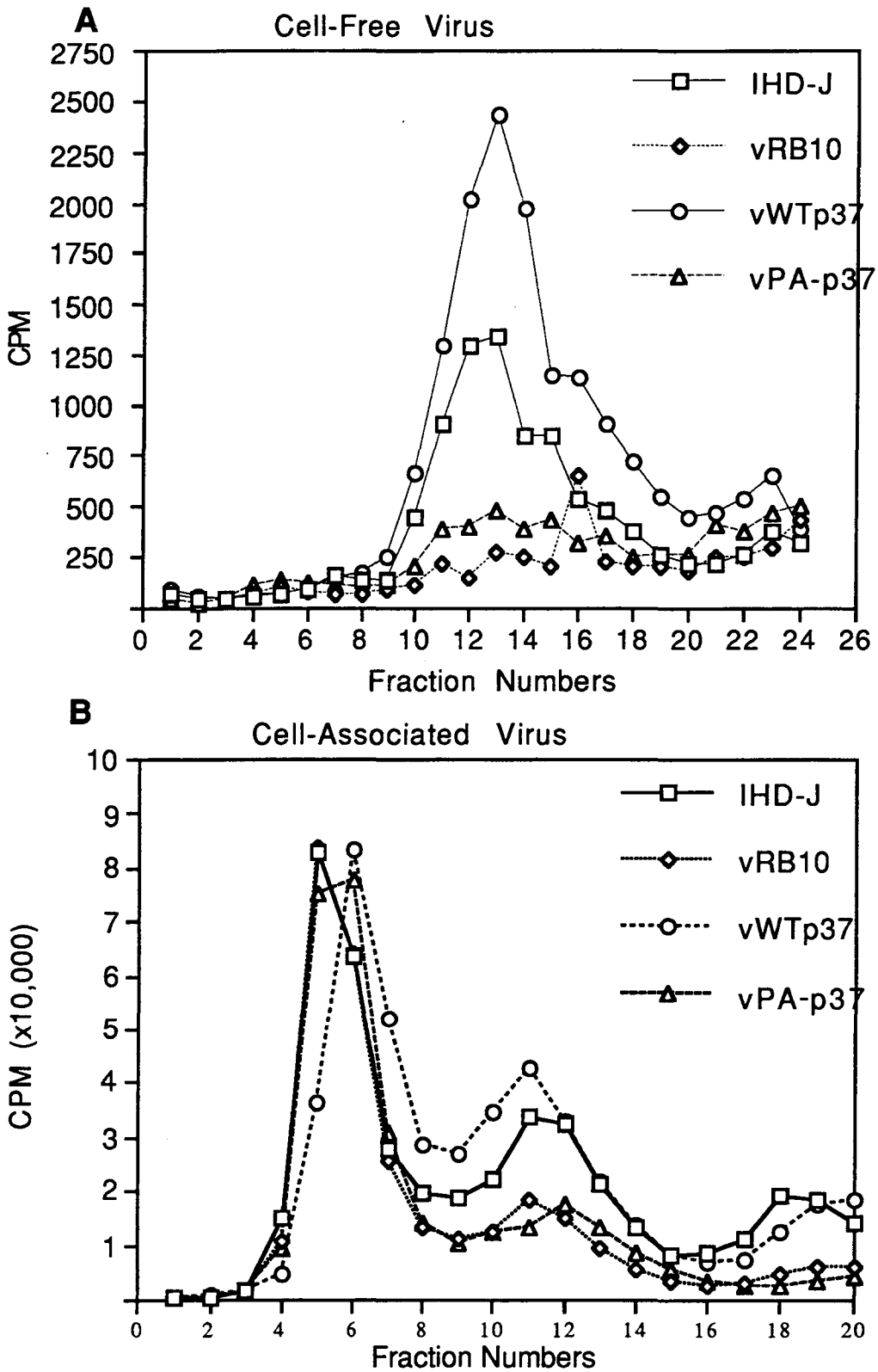
A
Cell-Free Virus
CPM
2750
2500
2250
2000
1750
1500
1250
1000
750
500
250
0 2 4 6 8 10 12 14 16 18 20 22 24 26
Fraction Numbers
IHD-J
vRB10
vWTp37
vPA-p37
B
Cell-Associated Virus
CPM (x10,000)
10
9
8
7
6
5
4
3
2
1
0 2 4 6 8 10 12 14 16 18 20
Fraction Numbers
IHD-J
vRB10
vWTp37
vPA-p37

Figure 3.5

(1.22-1.24 g/ml). Detection of enveloped virions above background was possible in vRB10-or vPA$^-$p37-infected cells, and both released a small amount of virus into the culture medium with buoyant densities characteristic of EEV (Figure 3.5A) but in both cases they were drastically reduced from the normal levels. A small amount of IMV was detectable in the culture media of all viruses at 24 hpi and was probably due to cell lysis.

These experiments indicate that vPA$^-$p37 is defective in the production and release of enveloped virions. vPA$^-$p37 does not display an intermediate phenotype, suggesting that palmitylation is absolutely necessary for p37 function regarding envelopment and release of virions.

3.3.3 EM Examination of vPA$^-$p37-Infected Cells

The morphogenesis of VV has been extensively characterized by transmission EM (21, 43, 79, 93, 94). The production of IMV occurs in discrete perinuclear foci termed viroplasm, virosomes or virus factories. At approximately 4 hpi the first membrane crescents appear in the virus factories and are most likely derived from the membranes of the intermediate compartment or vesicles from it. The membrane crescents become closed circles as the core components are loosely packaged within. Then the core condenses to the characteristic bi-concave or "dumbbell" shape and the virion acquires a brick-like shape. At this time it is possible to see by EM that the virion is wrapped by a single membrane. The IMV particle is then targeted to the membranes of the TGN. Upon budding through the compartment, two additional membranes are acquired, forming IEV. The formation of IEV is dependent upon p37 as well as numerous other VV-encoded proteins. Since vPA$^-$p37 appears defective for the production and release or enveloped virions, we examined infected cells by

transmission EM to determine the stage at which envelopment was arrested.

BSC_{40} cells were infected with virus at a moi of 10. At 16 hpi the cells were treated as outlined in Materials and Methods to prepare them for transmission EM. Upon comparing IHD-J and vRB10, two differences were noted: 1) While IHD-J virions had disseminated throughout the cell to the periphery (see vWTp37), vRB10 virions remained concentrated in perinuclear clusters distinct from, but near the virus factories (Figure 3.6). 2) At higher magnification it was not difficult to detect IHD-J virions in association with intracellular membranes, in the process of being enveloped by them, and fully enveloped as IEV, but all vRB10 virions had a distinct IMV morphology. As expected, vWTp37 was indistinguishable from IHD-J at high and low magnifications due to restoration of the wild-type F13L to the vRB10 genome. Within $vPA^{-}p37$-infected cells, only IMV could be observed, and like vRB10, most virions were concentrated in perinuclear clusters. It seems then, that palmitylated p37 is necessary not only for envelopment of IMV to form IEV, but also for movement away from the perinuclear location of virion production.

3.3.4 Fluorescent Microscopic Analysis of the Actin Stress Network and Localization of VV Antigens

Vaccinia uses actin polymerization for intracellular motility and projection outside the cell, presumably into neighboring uninfected cells. This is observed by fluorescent microscopy as a reorganization of the cellular actin stress network to form thick actin tails up to 0.74 μM in length that are tipped with a single IEV particle (18). Drugs or mutations that block the production of IEV also prevent the nucleation of actin tails (17) so we would expect $vPA^{-}p37$ to be defective for this process. Additionally, by transient expression of a

Figure 3.6. Electron microscopy of infected cells. Thin sections of BSC_{40} cells infected with virus as indicated above the electron micrographs were examined by transmission electron microscopy. Note virion clusters in vRB10- and vPA-p37-infected cells (16,400X magnification) while vWTp37 virions are well disseminated throughout the cell. Note also envelopment of vWTp37 virions by intracellular membranes (73,000X magnification). Original magnification is indicated to the left of the micrographs.

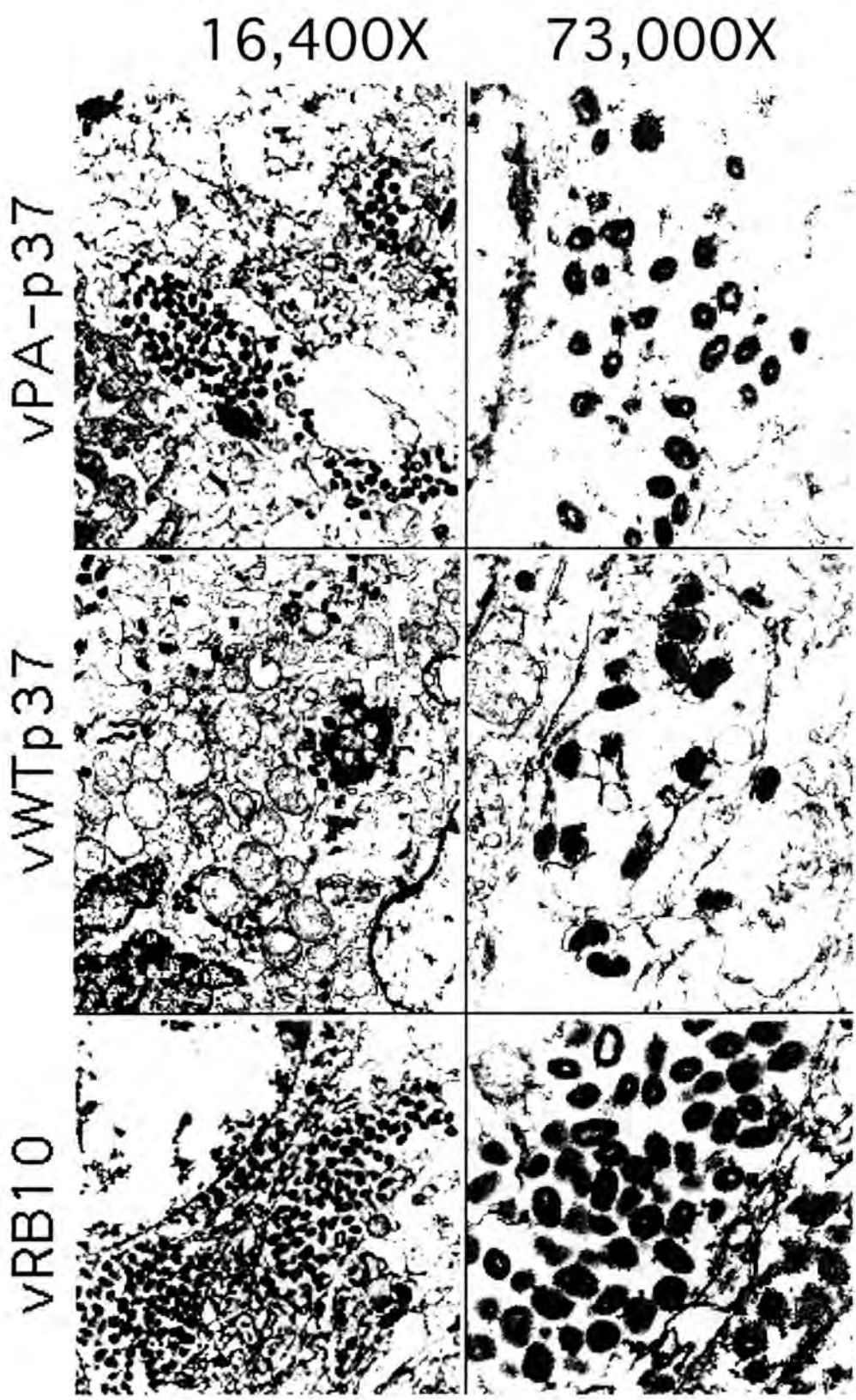

Figure 3.6

nonpalmitylated p37 within infected cells, we have demonstrated using immunofluorescent microscopy and subcellular fractionation that the mutated protein is at least partially soluble (34). Transient expression results in the overexpression of the protein in some cells while in others it is not expressed. Altered expression levels may have resulted in discrepancies between the wild-type protein and the mutant regarding localization. As an extension of our previous work, we repeated our assays for p37 localization using the recombinant viruses vWTp37 and vPA$^-$p37, which express p37 at levels indistinguishable from the wild-type IHD-J.

BSC_{40} cells were infected with virus at a moi of 10 for 8 h. The cells were fixed and stained with polyclonal sera to p37 and L1R, an IMV membrane-associated protein, and fluorescent secondary antibodies. F-actin was stained by using fluorescein-labeled phalloidin. Confocal fluorescent microscopy was used to observe localization of VV antigens and polymerized actin. Mock-infected cells had very little background fluorescence specific for the VV antigens while maintaining an intact actin cytoskeleton (Figure 3.7). At 8 hpi, both IHD-J and vWTp37 had completely reorganized the actin cytoskeleton. Numerous thick actin tails were visible and appeared to be tipped with virions based on p37 and L1R localization. Since it is known that when virion-associated, p37 is specific for the enveloped forms of the virus (IEV, CEV and EEV), it is likely that these represent IEV. The IMV outer membrane protein, L1R is somewhat concentrated in the perinuclear region of IHD-J- and vWTp37-infected cells, but is also distributed throughout the cytoplasm in a slightly punctate pattern. On the other hand, p37 is distinctly punctate throughout the cytoplasm of IHD-J- and vWTp37-infected cells as would be expected for a TGN-associated protein. Within vRB10-infected cells the actin cytoskeleton has been broken down but not reorganized into the thick filaments typical of the wild-type virus. Based on the localization of L1R, which is concentrated near the nucleus with little staining in the cytoplasm, virions appear

Figure 3.7. Immunofluorescent microscopy of infected cells. The localization of VV antigens, p37 and L1R, and F-actin were determined by indirect fluorescent labeling and confocal microscopy. The fluorescently labeled antigens are indicated at the top of each column and the infecting viruses are indicated to the left of each row. All images were captured through a 100X magnification oil immersion objective. Images of F-actin staining were zoomed 1.5X. Thick actin-containing microfilaments are indicated by arrows.

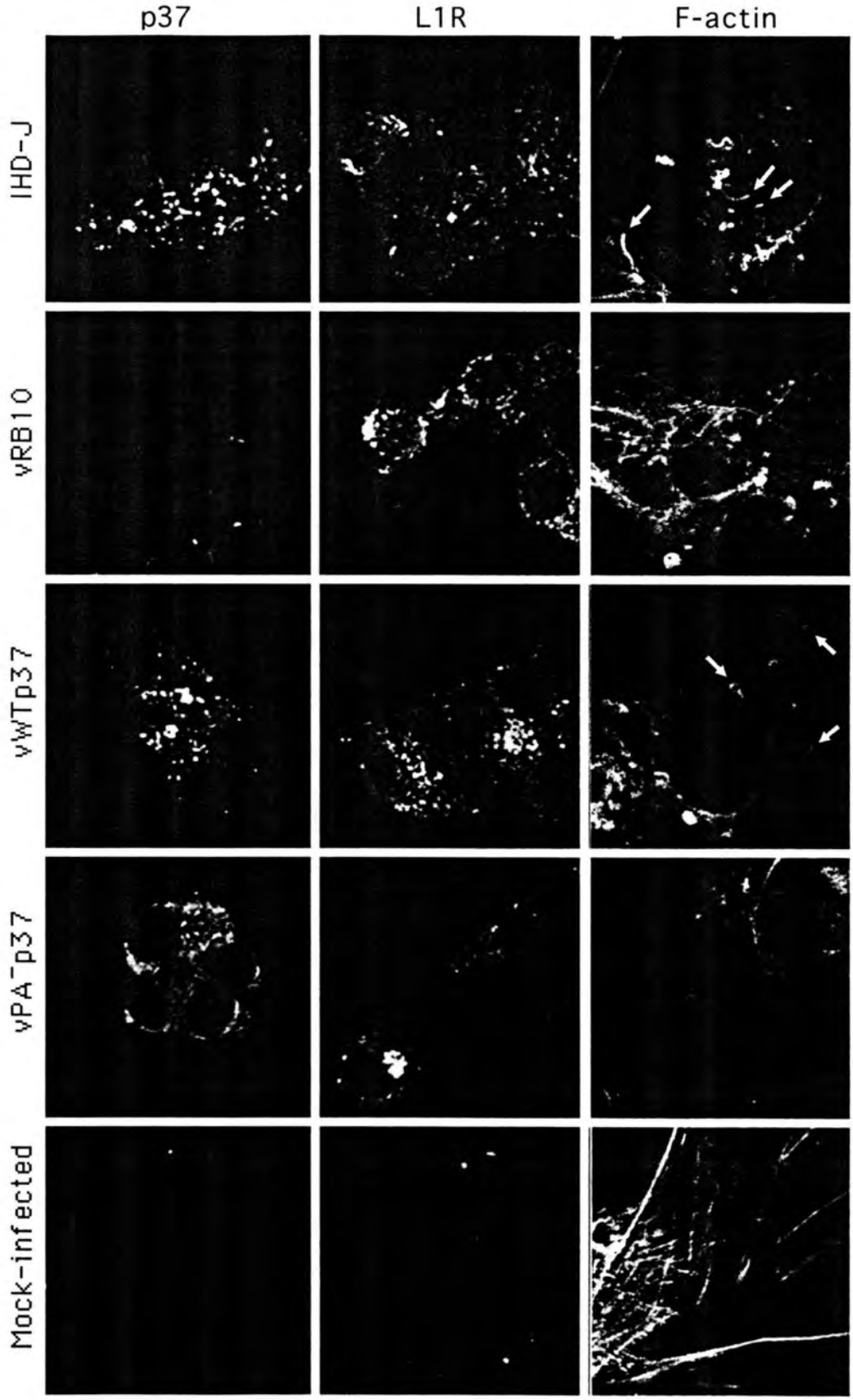

Figure 3.7

to have remained near the nucleus, in agreement with our EM observations. As expected, p37-specific fluorescence was not above background due to the deletion of F13L from vRB10. In agreement with our previous findings, p37 was distributed diffusely throughout the cytoplasm of vPA$^-$p37-infected cells, as would be expected for a soluble protein, while L1R-specific staining was concentrated near the nucleus, as was observed for vRB10. Like vRB10, the actin cytoskeleton was disassembled but not reorganized in vPA$^-$p37-infected cells.

3.3.5 Subcellular Fractionation of VV-Infected Cells

As we have discussed previously, the membrane interaction of p37 is mediated by the palmitate moiety. This was first demonstrated by Schmutz *et al* (91) and confirmed by site-directed mutagenesis and transient expression analysis of a nonpalmitylated p37 (34). Within infected cells, p37 is targeted to the membranes of the TGN and co-localizes with other EEV-specific antigens (84). If cellular membrane fractions containing p37 are treated with neutral hydroxylamine, cleaving palmitate from p37, then p37 becomes soluble. By indirect immunofluorescence and differential centrifugation subcellular fractionation, transiently expressed nonpalmitylated p37 behaves as a soluble protein and does not co-localize with other EEV-specific proteins as efficiently as the wild-type protein. We sought to confirm and extend these findings.

BSC_{40} cells were infected with virus at a moi of 10 for 16 h. The infected cells were then fractionated by differential centrifugation to yield a nuclear fraction (NP) and a cytoplasmic fraction (PNS) that was further fractionated to yield a particulate cytoplasmic fraction (P100) and a soluble cytoplasmic fraction (S100). In addition, the culture media (SUP) were clarified by a 100,000 x *g* centrifugation, concentrated by addition of

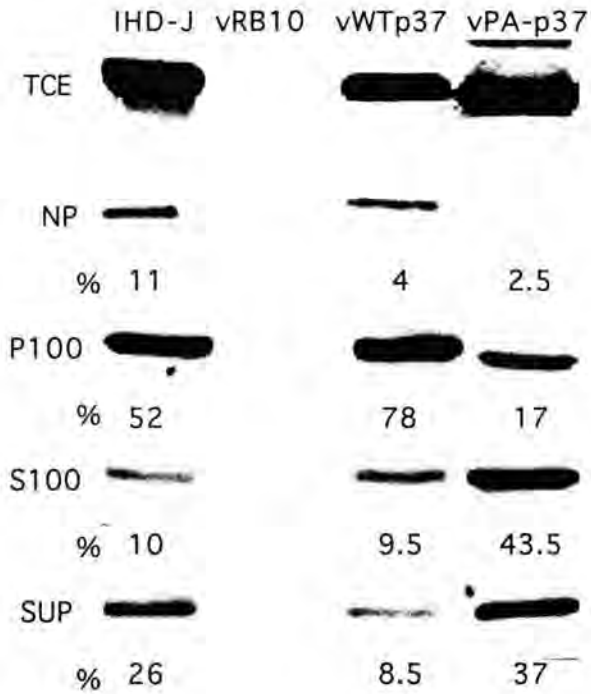

Figure 3.8. Subcellular fractionation of infected cells. Infected cells (as indicated above the topmost gel) were fractionated by differential centrifugation to yield a total cell extract (TCE), a nuclear fraction (NP), a particulate cytoplasmic fraction (P100) and a soluble cytoplasmic fraction (S100). The culture supernatants (SUP) along with the subcellular fractions were assayed for p37 by SDS-PAGE and immunoblots developed by chemiluminescence. Relative amounts of p37 were determined by film densitometry and the percent of the total (NP+P100+S100+SUP) for each virus are given below the gel of each fraction. Note the increased percentage of p37 in the S100 and SUP fractions of vPA$^-$p37.

trichloracetic acid and assayed along with the other fractions for p37 by SDS-PAGE and immunoblot. Following chemiluminescent development of the blots, the relative protein concentrations were obtained by film densitometry. While vPA$^-$p37 expressed p37 at similar levels as IHD-J and vWTp37 as determined by comparison of the total cell extracts (TCE), it was depleted from both the NP and P100 (Figure 3.8). The majority of vPA$^-$p37-

expressed p37 was found in the S100 and SUP fractions while very little IHD-J- or vWTp37-expressed p37 was found in the soluble fractions. This confirms our previous finding of increased solubility for p37 when not palmitylated.

3.3.6 Analysis of Virion-Associated p37

Schmutz *et al* (91) have demonstrated that p37 decorates the cytoplasmic face of the TGN and after virion envelopment is situated on the core-proximal face of the outermost envelope of EEV. It may be that p37 is EEV associated only by default due to TGN association but this is unlikely considering that p37 is necessary for envelopment of virus. A TGN-associated receptor for the IMV particle has not been identified, but seems likely to exist, as the IMV particle is specifically wrapped by TGN membranes. We considered the possibility that p37 is the TGN receptor for IMV and that it is not found to be IMV associated due to strong TGN interaction distal from the site of IMV production.

EEV and IMV were purified from cultures of metabolically labeled RK13 cells infected with either IHD-J, vRB10, vWTp37 or vPA$^-$p37. Equivalent amounts of EEV and equivalent amounts of IMV, as determined by radioactive counts per sample, were resolved by SDS-PAGE, blotted to nitrocellulose and probed with antibodies to p37, L1R and the 25-kDa core protein followed by secondary antibody incubations and chemiluminescent development (Figure 3.9B). As expected, p37 was found to be specific for EEV produced by IHD-J and vWTp37. We also found p37 to be vPA$^-$p37 EEV associated, but in relatively smaller amounts than that found on viruses encoding the wild-type p37. Surprisingly, vPA$^-$p37-produced p37 was also IMV associated, leading us to believe that it was the IMV receptor on the TGN. We also observed the L1R and the 25-kDA core protein to be both IMV and EEV associated as has been previously shown (71, 102). If p37

Figure 3.9. Analysis of virion-associated p37. Virus (as indicated above each gel lane) was purified, by CsCl gradients, from the culture medium of infected RK13 cells. (A) Resolution of EEV polypeptides by SDS-PAGE and autoradiography. Arrow indicates position of p37 in IHD-J and vWTp37 lanes. (B) Proteinase K treatment of purified EEV and IMV. Mock-treated or proteinase K-treated (as outlined in Materials and Methods) EEV and IMV were resolved by SDS-PAGE and blotted to nitrocellulose. The blots were sequentially probed for p37 and 25K then L1R, which are indicated to the left of each gel. Note the presence of undigested p37 in the proteinase K-treated IMV sample.

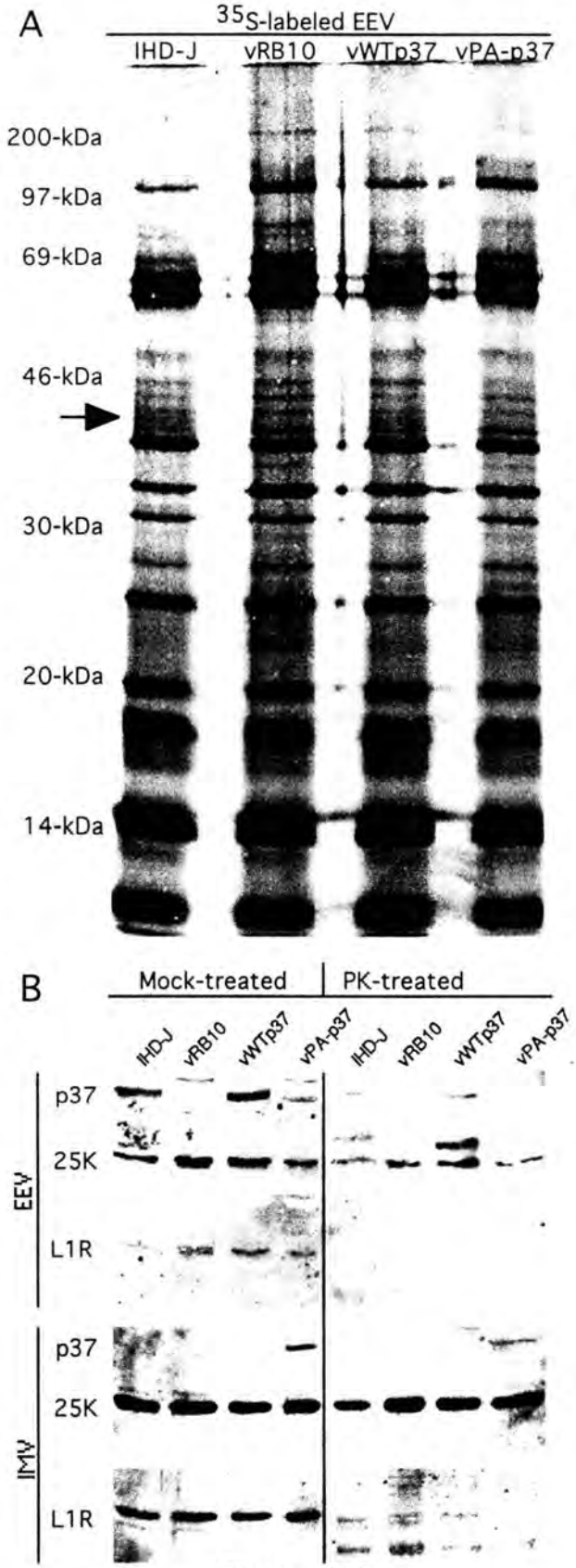
A
35S-labeled EEV
IHD-J
vRB10
vWTp37
vPA-p37
200-kDa
97-kDa
69-kDa
46-kDa
30-kDa
20-kDa
14-kDa
B
Mock-treated
PK-treated
IHD-J
vRB10
vWTp37
vPA-p37
EEV
p37
25K
L1R
IMV
p37
25K
L1R

Figure 3.9

is the TGN-associated ligand for IMV, then if soluble it should decorate the outside of IMV. To test this, equivalent amounts of EEV and equivalent amounts of IMV were treated with 2 μg/ml proteinase K (PK) for 1 h at room temperature to degrade any exposed proteins. Following PK treatment the reactions were stopped by addition of SDS-PAGE sample buffer and boiling for 3 min. The reaction mixtures were treated as above to detect p37, L1R and the 25-kDa core protein. The 25-kDa core protein was PK-resistant on both IMV and EEV while L1R was PK-sensitive on both IMV and EEV. The vPA$^-$p37 EEV-associated p37 was PK-sensitive while a portion of the IMV-associated p37 was PK-resistant. This suggests that p37 most likely is not exposed on the outer face of vPA$^-$p37 IMV but that it is protected within the core of the particle. Two major p37-derived degradation products were recognized by the p37 polyclonal antiserum. The larger fragment, of approximately 29 kDa, co-migrates with one of the three bands detectable by α–p37 in total cell extracts suggesting that some p37 is degraded by cellular or viral proteases in a manner similar to PK proteolysis. The smaller proteolytic product is detectable by α–p37 as a diffuse band running between 11 to 14 kDa.

3.4 Discussion

Palmitylation of p37 was first observed by Hiller and Weber (38). Since then the protein has been characterized extensively both biochemically and biologically. It is palmitylated and/or oleated (34, 65) on cysteines 185 and 186 of the 372-amino acid polypeptide. The fatty acyl modification serves as a membrane anchor, for without it the protein is soluble (34, 91). Within infected cells, p37 is targeted to the TGN (84) where it plays an important role in the envelopment and release of virions (9). The mutant virus, vRB10 does not express p37 and as a consequence produces very little IEV, CEV or EEV while producing normal amounts of IMV. Normally, VV reorganizes the actin cytoskeleton

using actin polymerization for intercellular and perhaps intracellular movement (17). Possibly because IEV are not formed, virus-tipped thick actin filaments are not found in vRB10-infected cells.

Recent findings suggest that p37 is a member of the phospholipase D superfamily based on the presence of a conserved motif (98). The H*X*K*XXXX*D motif (using the single letter amino acid code) and an additional conserved serine are only partially conserved in p37, but mutagenesis of any of the conserved residues results in a loss of function for the protein. These findings all indicate an important role for p37 in the late stages of the VV life cycle but do not entirely disclose the significance of the palmitate modification.

In this report we have described the construction and characterization of a VV mutant that expresses a nonpalmitylated p37 rather than the wild-type fatty acylated form. Because the known role of p37 is to mediate (along with other VV proteins) the envelopment of IMV, our assays were designed to examine that aspect of VV biology. In most assays, the nonpalmitylated p37 mutant virus (vPA$^-$p37) is indistinguishable from the F13L deletion mutant vRB10, suggesting that the fatty acyl moiety is indispensable for protein function.

Most often the purpose of palmitylation is membrane targeting and anchoring of proteins, but occasionally it serves another purpose (47, 54, 60). We considered the possibilities that palmitylation of p37 regulated protein-protein interactions or that the protein cycled between an inactive nonpalmitylated pool and an active palmitylated pool as a means of regulating protein function. It has not been conclusively demonstrated that p37 has any protein partners although it is known to co-localize with numerous VV proteins in infected cells (84). With what is known about p37 we can only conclude that palmitylation does not serve as a mediator of protein-protein interactions. Likewise,

palmitylation does not appear to serve as a regulatory mechanism for p37 function. As we have demonstrated by both subcellular fractionation and immunofluorescent microscopy, nearly 100% of the wild-type p37 is a component of the particulate fraction of cells, suggesting efficient membrane interaction. We have also demonstrated the protein-membrane interaction to be dependent on palmitylation as well as being necessary for p37 function. If palmitylation served as a regulatory function we would expect a significant fraction of the protein to be nonpalmitylated. Instead, we find that less than 1% of the protein is not palmitylated (34). It seems that the sole purpose of p37 palmitylation is to target and anchor the protein in the membranes of the *trans*-Golgi network. By our analyses presented here it is apparent that p37 interaction with the TGN is absolutely necessary for function.

In their analysis of p37, Baek *et al* purified bacterially expressed p37 and tested it for lipase activity, which they found. We have not observed palmitylation of p37 in bacterial cells. This suggests that palmitylation is not necessary for enzymatic activity and that the defect in the nonpalmitylated p37 is solely targeting. Considering our findings along with the findings of Baek *et al* and Sung *et al,* it appears that the lipase activity of p37 is required for the formation of IEV and that it must be specifically localized to the TGN for its activity to be biologically relevant. The *in vivo* substrates for this enzyme are unknown.

In summary, we have demonstrated palmitylation of p37 to be necessary for targeting p37 to the membranes of the TGN. The association of p37 with the TGN membranes is an absolute requirement for the function of p37. The primary biological activity of p37 is best demonstrated by the F13L deletion mutant vRB10, which is deficient for all aspects of envelopment and release of virus, while producing normal amount of IMV. When the wild-type F13L sequence is restored to the vRB10 genome, the mutant is rescued. But when the mutated F13L sequence, encoding a nonpalmitylated p37, is inserted into vRB10, the resulting

recombinant virus is indistinguishable from vRB10 in the assays described here. The lack of an intermediate phenotype suggests a complete loss of function for the protein.

Vaccinia virus continues to stand out as a uniquely capable model system for the analysis of mammalian protein processing. Besides acylation, VV polypeptides are subject to proteolytic processing, glycosylation, phosphorylation, ADP-ribosylation, disulfide cross-linking (103) and sulfation (65). We have yet to decipher all the intricacies of these modifications in VV or eukaryotic systems and palmitylation of proteins is one of the least understood of these processes. Perhaps exploitation of the VV system will allow us to examine not only various functions of protein palmitylation but to identify factors mediating the process, including those responsible for the modification reaction and molecular properties of the modified protein.

Chapter 4

Conclusions

Douglas W. Grosenbach

6 pages

4.1 Significance of the Research

The value of this work may be appreciated from two separate perspectives: as it relates to vaccinia virus biology, and to palmitylation of proteins in general. The series of events leading to the production of infectious virions from VV-infected cells is complex and understood only at the macromolecular level. The specific role of individual proteins is often unknown. By our study of protein palmitylation in VV-infected cells we have contributed to the understanding of intracellular and virion targeting of proteins as well as the process of virion envelopment and release. Palmitylation of proteins itself is not well understood. Although many palmitylproteins have been identified, as have their sites of modification, it has, to this point, been difficult to predict which proteins are palmitylated and on which residues. Through our efforts we have been able to predict not only which VV proteins are palmitylated, but also their sites of modification.

Research on VV continues to be significant for a number of reasons. First, VV itself is a pathogenic virus, closely related to smallpox. Although smallpox has been eradicated it has not been destroyed. In addition to the small amount of virus reserved for research purposes, the possibility exists that it may be (or has been) mass produced as a biological weapon. Research on VV biology provides insight for the treatment of smallpox should an outbreak occur. Second, VV is also used as a vaccine and a transient gene therapy vector. Recombinant DNA technology has allowed the development of VV beyond its traditional use as an anti-smallpox vaccine. To ensure the efficacy and safety of the vaccines/vectors, virulence mechanisms and the means to attenuate them must be understood. Third, VV is widely used as a transient expression vector. One of the reasons for its popularity is that proteins expressed by recombinant VV are processed as they would be in their native systems. The disadvantage to using

VV for transient expression is that the virus is cytopathic, resulting in cell death soon after infection. Research on VV must continue if its potential as a transient expression vector is to be realized

By our work we have demonstrated that palmitylation alone is sufficient to target a protein (p37) to its proper intracellular membrane-bound compartment. The loss of the targeting signal on p37 is debilitating for the virus resulting in an attenuated phenotype in tissue culture systems. This in itself highlights that the virus, as a result of its complexity, is dependent on numerous cellular processes. Any one of these processes may serve as a target for inhibiting virus replication. This is not to suggest that palmitylation of VV proteins would serve as an appropriate target for anti-viral therapeutics. In fact, the opposite is most likely true. Inhibition of protein palmitylation would likely be lethal for most cells, not just the virus. Since VV is easily manipulated at the genetic level, it would be possible though, using the research presented here, to construct recombinant/mutant viruses engineered to be deficient for certain processes and thus generate attenuated vaccines/vectors.

Palmitylation of proteins occurs in numerous if not all mammalian cell types. Many mammalian viruses encode at least one palmitylprotein as well. The significance of the modification is not always understood. The use of VV has allowed access to numerous palmitylproteins in an easily manipulated system. The ability to work with these proteins in a limited environment permitted the definition of a small number of biochemical characteristics that specify palmitylation of proteins. Extending from the work of others, analyzing substrates for palmitylation in other systems, we were able to define a primary structural motif in proteins specifying palmitylation. The motif is defined here as Hydro*1-12*AACA*, where "Hydro*" represents a hydrophobic element followed by a 1- to12-amino acid spacer. The spacer region precedes the palmitylated cysteine (C), which is often

surrounded by aliphatic (*A*) amino acids. Analysis of VV proteins for this motif led to the identification of seven of the nine VV-encoded palmitylproteins (if the COP strain hemagglutinin protein is counted). The sites of modification for five of the seven known palmitylproteins were accurately predicted simply by analysis of cysteine residues that fell within the motif.

The ramifications of this work extend beyond VV biology. As stated before, VV proteins are subject to numerous modifications typical of mammalian cell proteins and are processed in an authentic manner. The use of VV as a model system should allow a greater understanding of cellular palmitylproteins.

4.2 Suggestions for Future Research

VV-encoded palmitylproteins are all specific for the TGN-enveloped forms of the virus. Many of these proteins have been demonstrated to be involved either in the envelopment and dissemination of enveloped virions or in the infectivity of released enveloped virions. It will be of interest to determine the role, if any, of the A22R protein in the process of envelopment and release. Mutant viruses should be constructed which either do not express A22R or repress its expression unless induced. These viruses could then be compared to wild-type viruses in the same assays employed to characterize the nonpalmitylated p37 mutant vPA$^-$p37. The newly discovered VV-encoded palmitylproteins, as well as the previously known but uncharacterized VV palmitylproteins, A22R, A33R, A36R, A56R and B5R, offer the unique opportunity to study biological function of palmitylation on numerous proteins in a single, easily manipulated system. Most of these proteins are transmembrane proteins, so the function of the palmitate moiety is not so easily predicted. For some transmembrane proteins, palmitylation is dispensable for function while on others it is absolutely necessary.

It is not clear why the hemagglutinin protein (A56R) from the COP strain of VV would be palmitylated while the same protein, expressed from the WR and IHD-J strains, is not. The ability of these viruses to agglutinate red blood cells should be compared. Then, perhaps, viruses should be constructed which express the hemagglutinin from another strain and its virulence compared to the wild-type virus. All strains of variola (smallpox) as well as monkeypox contain a cysteine residue in the homologous position as the palmitylated cysteine in the COP hemagglutinin. It is possible that palmitylation of the hemagglutinin is a virulence factor for these viruses. Although it is not possible to work with smallpox, the ability to exchange these different genes between strains of VV should answer a few of the questions about the role of the palmitate moiety on the hemagglutinin.

The 14- and 17-kDa palmitylproteins are still unidentified. Our inability to predict which ORFs encode these proteins only highlight that our palmitylation motif is not perfect. More work must be done to determine what additional criteria should be included in screening for palmitylproteins. Factors that should be considered are; subcellular localization of the modification reaction, temporal regulation of the reaction, membrane orientation of the target protein, identification of enzymes or factors required for the reaction, and refinement of the primary structural motif.

Perhaps the most interesting work that needs to be performed is the determination of function for all of these proteins. The process of VV envelopment not only serves as a model system for protein:protein interaction and protein:membrane biochemistry, but is an important aspect of VV virulence. If VV is to be further developed as a vector/vaccine, its virulence mechanisms must be understood. An understanding of the role that these proteins play in the VV life cycle will likely provide numerous targets for attenuating the virus. To date, there

is no convincing evidence for protein:protein interaction by any of these proteins. Yet, these proteins all co-localize within infected cells, and are all involved in the envelopment of IMV. Perhaps the yeast two-hybrid system could be used to identify protein partners in the process of IMV envelopment.

Although the work presented in this thesis solves a number of scientific problems, it provides the basis for additional research that is perhaps of greater biological significance. Using this work as a foundation, scientists may now address the problem of protein palmitylation by a directed approach. The information provided here should allow the identification of new palmitylproteins and narrow the search for their sites of modification. Only then can the biological role of the modification be ascertained.

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